MATRIX METALLOPROTEINASES IN EXPERIMENTAL COLONIC HEALING

Thesis submitted for the degree of
Doctor of Philosophy
to the
University of London

by

Domingos Lourenço Penna Lacombe MD

Universidade Federal do Rio de Janeiro

Department of Surgery,
Faculty of Clinical Sciences,
University College London Medical School.
ABSTRACT

Matrix metalloproteinases in experimental colonic healing

Indirect evidence has suggested that collagenase, an enzyme that specifically degrades collagen is responsible for colonic dehiscence. This protease is only one member of a family of enzymes, the matrix metalloproteinases (MMPs), which between them degrade all the components of the extracellular matrix. The aim of this thesis is to investigate the distribution of MMPs and their inhibitor, tissue inhibitor of metalloproteinases, TIMP-1, in colonic anastomosis in rabbit models using immunohistochemistry.

Anastomotic healing in three different conditions has been investigated following; (i) transection of normal colon; (ii) colonic obstruction for 24h then resection; and (iii) ischaemia for 12h then resection; all carried out in the distal colon. To study different phases of the healing process, tissue from rabbits killed 12h, 1, 3 and 7 days following anastomosis was examined by indirect immunofluorescence. Sections were stained with haematoxylin and eosin and with antisera to collagenase, stromelysin, gelatinase and TIMP-1.

In normal healing the distribution of the MMPs was limited to the suture line with TIMP-1 always present at the same site. In an anastomosis following ischaemia MMPs and TIMP-1 were not restricted to the suture line but spread out proximally and distally. In an anastomosis following obstruction a similar pattern was observed but there was also extensive expression of MMPs beyond the anastomotic segment involving the entire colon. The distribution of MMPs was related to the extent of histological damage with extracellular MMPs, mainly collagenase earlier and stromelysin later after injury usually co-localised with TIMP-1.
In the normal healing model the co-existence of TIMP-1 with the MMPs demonstrated a controlled remodelling healing process. Lack of TIMP-1 in relation to MMPs, in the early period after anastomosis, suggests that uncontrolled degradation of the matrix may occur. This may be of relevance in obstruction and ischaemia where the wider distribution of MMPs with relative absence of TIMP-1 persists for longer.
TABLE OF CONTENTS

Abstract 2
Table of contents 4
List of Tables 7
List of Figures 8
Abbreviations 12
Acknowledgements 14
Dedication 15
Statement of originality 16

CHAPTER 1
INTRODUCTION 17

1.1 Historical development of colonic anastomosis 18
1.1.1 Sutures 20
1.1.2 Surgical techniques 21

1.2 Wound healing 25
1.2.1 Description of colonic healing 25
1.2.2 Biochemical aspects of wound healing 28

1.3 Colonic anastomosis dehiscence 29
1.3.1 Clinical studies 29
1.3.2 Experimental studies 35

1.4 Extracellular matrix and basement membrane 43
1.4.1 Collagen 44
1.4.1.i Molecular structure 45
1.4.1.ii Genetic types of collagen 46
1.4.1.iii The fibrillar collagens 48
1.4.1.iv Collagen biosynthesis 50
1.4.1.v Collagen degradation 52
1.4.2 Proteoglycans 53
1.4.2.i Extracellular proteoglycans 54
1.4.2.ii Cell surface proteoglycans 57
1.4.2.iii Intracellular proteoglycans 58
1.4.2.iv Biosynthesis and degradation of proteoglycans 58
1.4.3 Glycoproteins 59
1.4.3.i Fibronectin 59
1.4.3.ii Laminin 61
1.4.4 Elastin 61
1.5 Matrix metalloproteinases
1.5.1 The structure of metalloproteinases
1.5.2 Synthesis and secretion of metalloproteinases
1.5.3 Inhibition of matrix metalloproteinase activity
1.5.4 Connective tissue degradation by matrix metalloproteinases

1.6 Animal models of colonic anastomosis
1.6.1 The rabbit model
1.6.2 Suturing material and technique
1.6.3 Intestinal obstruction and colonic anastomosis
1.6.4 Intestinal ischaemia

CHAPTER 2

PURPOSE AND PLAN OF THE STUDY

CHAPTER 3

MATERIAL AND METHODS

3.1 Materials
3.2 Methods
3.2.1 Operative technique for colonic anastomosis
3.2.2 Sampling and preparation of tissue
3.2.3 Histological examination
3.2.4 Immunolocalisation
3.2.5 Histochemistry

CHAPTER 4

DEVELOPMENT OF MODELS

4.1 Normal healing model
4.1.1 Sham operation

4.2 Intestinal obstruction model
4.2.1 Development of the obstruction model
4.2.2 The obstruction model
4.2.3 Ring resection and anastomosis
4.2.4 Release of obstruction without anastomosis
4.2.5 Placement of a large ring

4.3 Ischaemic model
4.3.1 Development of the ischaemic model
4.3.2 The model of ischaemic bowel
CHAPTER 5

RESULTS

5.1 Normal healing model
5.1.2 Tissue from sham operations
5.1.3 Tissue from anastomotic operations
5.1.4 Summary of results in the normal healing model

5.2 Obstruction model
5.2.1 Tissue from the obstruction site (ring site)
5.2.2 Tissues from animals with anastomosis
5.2.3 Placement of a large ring
5.2.4 Summary of results in the group of obstruction and anastomosis
5.2.5 Tissues from animals without anastomosis
5.2.6 Summary of results in the group of obstruction without anastomosis

5.3 Ischaemic model
5.3.1 Ischaemic segment
5.3.2 Tissues from animals after resection and anastomosis
5.3.3 Summary of the results in the ischaemic model

CHAPTER 6

DISCUSSION

6.1 Introduction
6.1.1 Methodology

6.2 Normal healing model

6.3 Obstruction model
6.3.1 Obstruction only
6.3.2 Obstruction and anastomosis

6.4 Ischaemia model
6.4.1 The ischaemic segment
6.4.2 Ischaemia and anastomosis

6.5 Comparison of the models

6.6 The interplay of MMPs and TIMP-1
6.6.1 Introduction
6.6.2 Collagenase
6.6.3 Stromelysin
6.6.4 Gelatinase
6.6.5 TIMP-1

6.7 Possible regulatory mechanisms

6.8 Conclusions

References
List of Tables

1. I Collagen types

5. I Distribution of MMPs and TIMP-1 after anastomosis in the normal healing model

5. II Distribution of MMPs and TIMP-1 in the animals killed after 24 h of obstruction only

5. III Distribution of MMPs and TIMP-1 in the animals killed 24 h after releasing the obstruction

5. IV Distribution of MMPs and TIMP-1 in the animals killed 3 days after releasing the obstruction

5. V Distribution of MMPs and TIMP-1 immediately after formation of an anastomosis following an obstruction

5. VI Distribution of MMPs and TIMP-1 12 h after resection and anastomosis following obstruction

5. VII Distribution of MMPs and TIMP-1 24 h after resection and anastomosis following an obstruction

5. VIII Distribution of MMPs and TIMP-1 3 days after resection and anastomosis following an obstruction

5. IX Distribution of MMPs and TIMP-1 7 days after formation of an anastomosis following an obstruction

5. X Distribution of MMPs and TIMP-1 in the ischaemic segment after 12 h of ischaemia

5. XI Distribution of MMPs and TIMP-1 12 h after resection and anastomosis following ischaemia

5. XII Distribution of MMPs and TIMP-1 24 h after resection and anastomosis following ischaemia

5. XIII Distribution of MMPs and TIMP-1 3 days after resection and anastomosis following ischaemia
List of Figures

1.1 Domain structure of the matrix metalloproteinases

4.1 Diagram of blood supply to distal colon in rabbit showing location of transection and anastomosis

4.2a Tissue segments taken for immunohistochemistry and H&E in the normal healing model

4.2b Tissue segments taken for immunohistochemistry and H&E in the obstruction + anastomosis group

4.3a Diagram of distal colon in rabbit showing site of ring and resection used in obstruction model

4.3b Figure showing the ring in place

4.3c Figure showing the obstructed bowel 24 h after placement of the ring

4.3d Photograph showing the resected segment containing the ring

4.3e The same segment after remove the ring and opening the bowel

4.3f Photograph showing an anastomosis 24 h after resection of the ring in the obstruction model

4.4a Tissue segments taken for immunohistochemistry and H&E in the obstruction only group

4.4b Tissue segments taken for immunohistochemistry and H&E in the ischaemic model

4.5a Photograph showing the normal blood supply to the distal colon

4.5b Aspect of the distal bowel after 2 h of ischaemia

4.5c View of the distal colon after 4 h of ischaemia

4.5d Photograph of the distal colon after 6 h of ischaemia

4.6a Histology of the bowel after 2 h of ischaemia

4.6b H&E staining of the ischaemic segment 4 h after tying the vessels

4.6c Section of the centre of the ischaemic segment 4 h after ischaemia

4.6d Section showing the tissue damage after 6 h of ischaemia

4.7 View of the necrotic segment after 12 h of ischaemia

4.8a,b Two H&E staining of the necrotic bowel 12 h after ischaemia

4.8c,d Details of fig 4.8b showing the muscle layer

4.9a,b Sections of the proximal ischaemic segment stained with H&E. a show the region near the proximal border and b show the region near the centre
4.9. c,d Sections of the distal ischaemic segment showing in c the region near the distal border and in d the region near the centre

4.10 Diagram showing the blood vessels tied to produce an ischaemic segment in the distal colon

5.1 Section showing the rabbit normal distal colon at the site of anastomosis

5.2. a H&E staining of the anastomotic segment 12 h after anastomosis in the normal healing model

5.2. b Diagram showing the distribution of MMPs and TIMP-1 12 h after transection and anastomosis

5.2. c,d Details of figure 5.2. a showing inflammatory cell infiltration

5.2. e,f Immunolocalisation of collagenase in the mucosa and muscle of the anastomotic segment 12 h after transection and anastomosis

5.2. g,h Sections at the same region of figure 5.2. e incubated with antiserum to gelatinase and stromelysin

5.3. a H&E stained section showing the anastomotic segment 24 h after transection and anastomosis

5.3. b Diagram showing the distribution of MMPs and TIMP-1 24 h after anastomosis in the normal healing model

5.4. a H&E stained section showing the anastomotic segment 3 days after transection and anastomosis

5.4. b Diagram showing the distribution of MMPs and TIMP-1 3 days after anastomosis in the normal healing model

5.5. a H&E stained section showing the anastomotic segment 7 days after transection and anastomosis

5.5. b Diagram showing the distribution of MMPs and TIMP-1 7 days after anastomosis in the normal healing model

5.5. c,d Details of figure 5.5. a showing inflammatory cell infiltration in the fibrous tissue

5.5. e,f Sections of the fibrous tissue at the anastomotic segment incubated with antiserum to gelatinase and stromelysin demonstrating intracellular enzymes

5.5. g Section of the mucosa at the same segment of figure 5.5. e incubated with antiserum to stromelysin showing extracellular secretion

5.6. a H&E stained section showing the anastomotic segment 12 h after resection of the segment containing the ring and anastomosis formation

5.6. b Diagram showing the distribution of MMPs and TIMP-1 in the anastomotic segment of figure 5.6. a
5.6. c,d Details of figure 5.6. a showing inflammatory cell infiltration

5.6. e,f Section of the mucosa of the segment of the figure 5.6. a incubated with antiserum to collagenase and TIMP-1

5.6. g,h Section of the mucosa of the segment of the figure 5.6. a incubated with antiserum to gelatinase and stromelysin

5.6. i,j Section of the segment of figures 5.6. a in the submucosa stained with antiserum which recognise Mr 95000 form of gelatinase

5.6. k Section of the segment of figures 5.6. a in the submucosa stained with antiserum which recognise Mr 72000 and Mr 95000 forms of gelatinase

5.7. a,b Section show typical histological abnormalities 12 h after resection of the ring and anastomosis formation

5.8. a H&E stained section showing the anastomotic segment 24 h after resection of the segment containing the ring and anastomosis formation

5.8. b Diagram showing the distribution of MMPs and TIMP-1 in the anastomotic segment of figure 5.8. a

5.8. c Detail of figure 5.8. a showing stitches surrounded by large number of inflammatory cells

5.9. a H&E stained section showing the anastomotic segment 3 days after resection of the segment containing the ring and anastomosis formation

5.9. b Diagram showing the distribution of MMPs and TIMP-1 in the anastomotic segment of figure 5.9. a

5.9. c,d Section of the mucosa of the segment of the figure 5.9. a incubated with antiserum to collagenase and TIMP-1

5.9. e,f Section of the mucosa of the segment of the figure 5.9. a incubated with antiserum to gelatinase and stromelysin

5.10. a H&E stained section showing the anastomotic segment 7 days after resection of the segment containing the ring and anastomosis formation

5.10. b Diagram showing the distribution of MMPs and TIMP-1 in the anastomotic segment of figure 5.10. a

5.11. a Section of Prox0 segment after 24 h of obstruction stained with H&E

5.11. b Diagram showing distribution of MMPs and TIMP-1 in the segment of figure 5.11. a

5.11. c,d Details of figure 5.11. a showing extensive mucosal damage and inflammatory cell infiltration

5.11. e Section of the segment of figures 5.11. a,c,d in the submucosa stained with antiserum which recognise Mr 72000 and Mr 95000 forms of gelatinase

5.12. a Section of Prox1 segment after 24 h of obstruction stained with H&E
5.12.b Diagram showing distribution of MMPs and TIMP-1 in the segment of figure 5.12.a

5.12.c,d Details of figure 5.12.a showing extensive mucosal damage and inflammatory cell infiltration

5.13.a Section of the mucosa of Prox0 segment 12 h after anastomosis formed after 12 h of ischaemia incubated with antiserum to stromelysin

5.13.b Section of the distal segment of the same animal of figure 5.13.a incubated with antiserum to TIMP-1

5.14.a H&E stained section showing the anastomotic segment 24 h after resection of the ischaemic segment and anastomosis formation

5.14.b Diagram showing the distribution of MMPs and TIMP-1 in the anastomotic segment of figure 5.14.a

5.15.a H&E stained section showing the anastomotic segment 3 days after resection of the ischaemic segment and anastomosis formation

5.15.b Diagram showing the distribution of MMPs and TIMP-1 in the anastomotic segment of figure 5.15.a
Abbreviations

Å - ångström
BAR - biofragmentable anastomosis ring
BM - basement membrane
C - centigrade
cm - centimetre
CNP30 - 1,1,1-trichloroethane and 1,2-epoxypropane
CO₂ - carbon dioxide
DPX - mounting medium
ECM - extracellular matrix
ESAF - endothelial cell stimulating angiogenic factor
F(ab) - monovalent antibody fragment
Fig - figure
FITC - fluorescein isothiocyanate
g - gram
Gly - glycine
h - hours
H&E - haematoxylin and eosin
HEPES - 4-(2-hydroxyethyl)-piperazine-ethane sulphonic acid
IgG - immunoglobulin G
ile - isoleucine
kDa - kilodalton
kg - kilogram
leu - leucine
M - molar
mg - milligram
min - minute(s)
ml - millilitre
mm - millimetre
mmHg - millimetres of mercury
MMP - matrix metalloproteinase
Mr - molecular weight
mRNA - messenger ribonucleic acid
μM - micromolar
nm - nanometre
NSS - normal sheep serum
PBS - phosphate buffered saline
PFU - perfusion units
PMN - polymorphonuclear leukocyte
SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis
pO2 - oxygen tension
RNA - ribonucleic acid
TIMP - tissue inhibitor of metalloproteinases
U.K. - United Kingdom
U.S.A. - United States of America
ACKNOWLEDGEMENTS

This thesis would not have been possible without the help of several members of the Department of Surgery, University College London Medical School.

I am sincerely grateful to Mr Paul Boulos, Reader in Surgery, who enrolled me into his research programme, for his guidance and support and for supervising the project.

A very special acknowledgement to Dr Felicity Savage who devoted considerable time to teaching me the scientific principles and techniques, for her constant help and advice and for her tolerance in supervising my laboratory work.

I thank Professor Michael Hobsley and Professor Irving Taylor for their constant interest in my project.

I am indebted to Dr J.J. Reynolds and Dr R.M. Hembry of Strangeways Research Laboratory, Cambridge for supplying the antisera, crucial for this work, and for availing us of their expertise whenever needed.

My sincere thanks to Dr Virginia Sams, Senior Lecturer in the Department of Histopathology who developed my skills in histopathology and advised on the histopathology of the models investigated in this thesis.

I am thankful to Marilena Loizidou for her constructive criticism, to Daniel Anthony who instructed me in the use of computers and to Abdul Gafur for his friendship and help with the photographs.

I am grateful to the Conselho Nacional de Desenvolvimento Cientifico e Tequinologico from Brazil and the Universidade Federal do Rio de Janeiro for their generosity in awarding me a grant that made it possible for me to study for a higher degree in the United Kingdom.
This thesis is dedicated to Dores who left her country and family to accompany me, without her it would be impossible to make my research; to Guilherme, Isabel and Bruno, their support during all these years out of home was the most precious all.
STATEMENT OF ORIGINALITY

The studies presented in this thesis were designed and personally carried out by the author. There has been no similar study on metalloproteinases in experimental colonic healing.

The animal models developed in this thesis were based on similar models but were modified for the purpose of the study. The immunohistochemical methods were devised by Dr R.M. Hembry of Strangeways Research Laboratory, Cambridge and were modified by the author for these rabbit colon studies.
CHAPTER 1

INTRODUCTION

The first account of a bowel wound, with subsequent death, appeared in the Bible (Judges 3: 16-25).

... and the fat closed over the blade,
so that he could not draw the dagger
out of his belly; and the dirt came out. ...  
And behold their lord was fallen down dead
on the earth.

Since, intestinal suture has gradually evolved and an anastomosis has become a standard and frequently needed operative procedure. However the risk of suture line breakdown and leakage remains a matter of uncertainty and controversy (Goligher et al 1970a, Scott-Conner and Scher 1987) and has initiated considerable research interest (Koruda and Rolandelli 1990). Multiple factors are responsible for anastomotic dehiscence and some unclarity still overshadows our understanding of the mechanisms involved. Indirect evidence has suggested that collagenase, an enzyme that specifically degrades collagen plays part in colonic dehiscence. Collagenase is only one member of a family of connective tissue degrading enzymes known as the matrix metalloproteinases (MMPs). The other members of the family are the gelatinases and the stromelysins and together they can degrade all the components of the extracellular matrix, but their involvement in colonic damage and repair has not been explored. The current work examines this aspect of what is probably a complex system and is aimed to investigate the possible role of the matrix metalloproteinases and their inhibitor, tissue inhibitor of metalloproteinases (TIMP) by examining their distribution in colonic anastomosis in an animal model.
1.1 Historical development of colonic anastomosis

Since ancient times spontaneous recovery from bowel wounds has infrequently been described probably because in rare cases peritoneal adhesion to the injured intestine prevents the leakage of contents into the peritoneal cavity. The first surgeon to advocate intestinal suturing was Celsus in his book "De medicina" edited in ancient Rome (Spencer WG, English translation, 1938). Celsus believed that if the wound was in the small bowel it could be left but if it was in the large bowel it should be sutured because spontaneous healing was unlikely to occur. Reviews by Kerr (1923) and Wangensteen (1978) have covered early accounts of the evolution of intestinal suturing.

In 1396 Lanfranck suggested suturing the viscera into the parietal peritoneum to encourage fistula formation into the skin surface. The earliest report of bowel excision and suturing was from Ramdohr in 1727. He excised two feet of gangrenous small bowel with its mesentery, then invaginated the proximal end of the bowel into the lumen of the distal segment and fixed it with a few sutures. The patient survived. Duverger in 1747 carried out the same procedure but rejoined the ends with a few sutures over a piece of animal trachea, before placing the bowel in the peritoneal cavity. The trachea was expelled in the stool on the twenty-first postoperative day. In 1812 Travers reported his experiments in dogs and horses showing that an intestinal wound could be repaired without the support of the parietal peritoneum provided that in suturing the bowel the visceral peritoneum was tightly approximated sealing the lumen completely before returning the gut into the abdomen. The Russian surgeon, Pirogoff, also employed intestinal suturing successfully during the Crimean war in 1856.

Lembert in 1826 described a technique for a single layer anastomosis that is still employed. He placed interrupted sutures, 6 to 8 mm apart, that passed through the serosa and the submucosa but omitted the mucosa, resulting in serosal inversion, as had been stressed by Travers in 1812. Czerny 1880 recommended suturing the bowel in two layers, the first through the full thickness of the bowel wall, usually of continuous
catgut or interrupted silk, to allow good apposition of the cut edges and to secure haemostasis. The second layer was an outer seromuscular or a Lembert stitch, often interrupted and of non-absorbable material to produce inversion and bring the peritoneal coats together. Halsted (1887) was the first to carry out a systematic study of intestinal repair with experiments in dogs demonstrating that the submucosa was the most important layer of the bowel in determining the anastomotic strength. The work of Halsted was based on the monograph "Experimental and clinical enquiry into the nature and treatment of wounds of the intestine" by Samuel D. Gross in 1843, who pointed out that the submucosa is the strongest coat of the intestine. Halsted showed that it is impossible to suture the serosa alone without piercing the submucosa and that stitches which penetrate the lumen are undesirable and are apt to be associated with adhesions. In spite of advocating the use of a submucosal inverting suture, Halsted drew attention to the dangers of folding-in an unnecessarily excessive intestinal wall because this increases the tissue bulk undergoing sloughing thus augmenting the risk of infection. Also tissue inverted into the intestinal lumen could obstruct the passage of faeces increasing the tension on the sutures (Halsted 1887). Gambee (1951) also counselled against excessive inversion and described a single layer suture that allowed a secured apposition of the cut edges of the bowel with minimal inversion.

The work of Travers, Lembert and Halsted established the fundamentals of bowel anastomosis at the end of the nineteenth century. However the majority of bowel wounds at that time were fatal irrespective of whether or not suturing was attempted (Dunphy 1970) and for this reason the prevailing view, especially in military circles until the early phases of World War I, was not to operate. It was not until the innovation of general anaesthesia in 1844 and the introduction of antisepsis by Lister in 1867 that the development of colonic surgery began, with further impetus from the discovery of antibiotics by Fleming in 1929.
Despite the work carried out in the nineteenth century showing the advantages of inverted anastomoses, Getzen in 1966 presented experimental evidence favouring everted anastomoses and these findings were confirmed by Hamilton 1967; Ravitch et al 1967; Healey et al 1967; Loeb 1967 and Buyers and Meier 1968. However other experimental work from Canalis and Ravitch 1968; Hargreaves and Keddie 1968; Mellish et al 1968; Singleton et al 1968; Gill et al 1969; McAdam et al 1969; Orr 1969 and Rusca et al 1969 supported an inverted suture technique. Trueblood et al (1969) using an experimental model of anastomosis in rats provided definitive evidence that the inversion suture, when properly performed, provides greater tensile strength, a diminished tendency to leak and far less peritoneal adhesions than the everted one. His histological studies showed that with the inverted technique the healing is more rapid with earlier restoration of mucosal continuity than with an everted suture. Goligher et al (1970b) instituted a controlled clinical trial of everting versus inverting suture in large bowel surgery that had to be terminated prematurely because inverted, were significantly better than everted suturing. In this trial a higher proportion of patients with the everted technique developed wound infection or peritonitis and faecal fistulation was much more frequent. The principles of Travers and Lembert (apposition of cut edges and serosa) and Halsted (inverting sutures piercing the submucosa) were then re-established and are still valid.

1.1.1 Sutures

Many different types of suture material are available and surgeons have searched for the ideal one. Halsted (1913) strongly advised the use of silk instead of catgut as did Whipple (1933) who after clinical and experimental studies comparing silk and catgut sutures in parallel rectus sheath incisions in rabbits showed that catgut caused a much increased leukocytosis and necrosis than silk, which produced a wound with a higher tensile strength. In clinical observations he found much less infection in the wounds closed with silk than those with catgut. The evaluation of suture material in
colonic anastomoses has expanded since the 1950s when many new suture materials became available and these will be discussed further in chapter 1.6.

1.1.2 Surgical techniques

Although the basic principles of intestinal anastomosis are undisputed there remains the problem of how to achieve the best result. Should the anastomosis be of one or two layers of sutures, a continuous or an interrupted stitch and which type of suture material should be used? The conventional two-layer anastomosis uses an inner full thickness continuous layer of absorbable sutures and an outer seromuscular layer of interrupted non absorbable sutures. The one-layer anastomosis is usually performed with interrupted sutures inserted through the serosal surface and brought out in the submucosal layer at the cut edge rather than through the full thickness of the bowel wall. A good approximation of the serosa, the muscle and submucosal layers with a minimum inversion of the mucosa, not included in the suture, is obtained by insertion of sutures very near the cut edge, resulting in a smooth anastomosis without occlusion of the lumen. A two-layer inverting anastomosis allows a good apposition of the cut edges of the bowel but reduces mucosal blood flow (Chung, 1987) and results in a larger degree of stenosis than the single layer technique (Sacko and Wangensteen, 1951, McAdams et al, 1970, Irvin and Edwards, 1973). Koruda and Rolandel (1990) reviewing experimental studies on the healing of colonic anastomoses concluded that single layer closure in colonic anastomoses will allow improved healing provided that the amount of tissue inverted by the suture line is minimised to avert compromising the mucosal blood flow and the luminal diameter irrespective of the suture material and the suturing method used.

By the end of the 19th century sufficient experience had been gained by abdominal surgeons to show that colonic anastomosis of the left colon was much more hazardous than an anastomosis elsewhere in the gastrointestinal tract, with a
considerable risk of leakage and infection. Therefore in addition to attention to the suturing technique that allowed adequate healing without leakage, means of reducing the risk of faecal contamination were sought.

Wangensteen (1978) records Murphy in 1892 as the surgeon who introduced a small circular metal button with two parts, the first ingenious device to avoid contamination of the peritoneum with an expedient anastomoses. Each part was inserted into a segment of the two hollow viscera to be approximated and fixed with a purse-string suture of catgut. The two buttons, male and female, were then pushed home: a spring held them firmly in position. A continuous over-and-over catgut suture or interrupted silk suture sufficed to terminate the operation. The button passed spontaneously distally into the intestine and was readily evacuated per rectum. The Murphy Button was widely used at the beginning of this century (Halsted 1922) and has recently been revived as the biofragmental anastomosis ring (Hardy 1985).

In 1984 Goligher reviewed colorectal surgery since the end of the last century. Bloch in 1894 was the first surgeon to carry out a sigmoid resection by an extraperitoneal technique which consisted of exteriorising the sigmoid loop containing the tumour without removing it and inserting a tube in the proximal limb to deflate the bowel. After the colon had become fixed to the abdominal wall the sigmoid colon was excised and some time later the resulting double-barrelled colostomy was closed with the help of an enterotome. Paul in 1895 and Mickulics in 1903 used similar methods with Paul resecting the exteriorised loop at the first operation and fixing two large glass tubes in the limbs of the projecting bowel. The second stage was the same as Bloch, in using an enterotome to destroy the spur between the two limbs of the bowel. The remaining fistula either closed spontaneously or was sutured. Using the extraperitoneal technique of delayed resection Mickulics reported a reduction in operative mortality from 42.9% for resection with primary anastomosis to 12.5%. The technique soon was very popular and many surgeons such as Rankin in 1926, Devine in 1931,
Lahey in 1932, Lloyd-Davies in 1945 broadened the scope of the operation for extended resections and for resecting tumours in the fixed segments of the colon after adequate mobilisation.

Another method that was developed to avoid the risk of infection after colonic anastomosis was the "aseptic" anastomosis. This attempted to resect and suture, delaying opening the bowel lumen until the anastomosis was almost complete. Usually two crushing clamps were used and the bowel divided between them. The two clamped bowel ends were opposed and turning the clamps away and towards the suture line, the anterior and posterior rows of Lembert sutures were inserted and tied. The clamps were then opened slightly and withdrawn and the defect in the row of seromuscular suture where the forceps had been placed was closed by placing few additional stitches. Palpating the anastomosis between finger and thumb separated the crushed edges and re-established the lumen at the site of the anastomosis. The technique reduced considerably the amount of soiling during the operation and was used by a large number of surgeons from the end of the 19th century to the first half of the 20th century (Halsted 1898, O'Hara 1900, Parker and Kerr 1908, Schoemaker 1921, Fraser and Dott 1924, Pringle 1924, Rankin 1928 and Wangensteen 1940).

A temporary caecostomy connected to a rubber tube for drainage has also been employed to "protect" the anastomosis by relieving tension in the colon and reducing the strain on the suture line (Cheever 1931, Wilkie 1934, Rowlands and Turner 1937). Caecostomy usually closed spontaneously on withdrawal of the tube 14 days later. More radical was Devine (1931) who formed a preliminary transverse colostomy two or three weeks prior to the operation and carried out irrigation via the colostomy and rectally to empty the left colon completely. The surgeon could then make a resection on a "collapsed, defunctioned and debacterialised" distal colon.
After World War II advances in general anaesthesia, a better understanding of fluid and electrolyte balance and the use of blood transfusion offered the surgeon much better operating conditions. This allowed a more careful and meticulous operative technique when performing an anastomosis with minimal contamination. The introduction of sulphonamides and antibiotics in the 1940s had a considerable impact on colorectal surgery as they could be used both for systemic therapy and for sterilising the colon. The possibility of intestinal antisepsis and treatment of peritoneal infection encouraged surgeons to proceed with immediate resection and intraperitoneal anastomosis avoiding a colostomy and a delayed anastomoses. This approach has become the practice when it has been shown to be as safe as the staged procedures (Lloyd-Davies 1953).

The recent development of stapling instruments described by Goligher (1984) particularly the automatic circular stapler has enabled surgeons to carry out very low inverting in colorectal and coloanal anastomosis. The gastrointestinal stapling instrument was invented by the Hungarian surgeon, Hurtl and the instrument manufacturer Fischer and subsequently modified and improved by von Petz (Goligher 1984). Stapling devices were perfected and reintroduced by the Russians in the 1950s (Ravitch 1984). When properly used, staplers produce anastomoses comparable in reliability to hand-sewn ones and in difficult anatomical situations such as in pelvic surgery the instrument makes end to end colorectal and coloanal anastomoses much easier to perform and there is universal acceptance of its use in sphincter-saving procedures. However despite the improvements in suture material, surgical techniques and in bowel preparation, anastomotic dehiscence does still occur, sometimes for no apparent reason.
1.2 Wound healing

The healing process has been much more extensively studied in skin and fascia than in other less homogenous tissues, such as the colon, however it is generally assumed that the process is the same in all soft tissues (Herrmann et al 1964). Essentially wounds of soft tissues heal by a process of connective tissue repair and the formation of a fibrous scar. Two types of repair are seen in soft tissue wounds. Primary wound healing occurs in incised wounds when the edges of the wound are apposed, and this is the type of repair encountered in the majority of surgical wounds. Secondary wound healing occurs in wounds associated with tissue loss or when the wound edges are widely separated. This method of repair is mainly found in contaminated and burn wounds. Although the basic features are the same in bowel anastomoses, healing in the colon is disturbed by the large numbers of bacteria and bulky faeces in the bowel lumen and by a rapid turnover of collagen following injury when as much as 40% of normal colonic collagen in the anastomotic region is lost by the third day (Cronin et al 1968). These factors are at least partially responsible for an appreciable morbidity and mortality due to failure of the healing process following large bowel anastomosis.

1.2.1 Description of colonic healing

Histological observations show that three phases can be identified in intestinal healing which correlate with biochemical changes associated with wound healing (Hermann et al 1964):

Phase I - 0 to 4 days. Lag phase - inflammation and oedema.

Phase II - 3 to 14 days. Logarithmic phase - proliferative fibrous repair.

Phase III - 10 to 180 days. Stationary phase - reorganisation and remodelling.
Phase I involves an acute inflammatory reaction, accompanied by diffuse oedema and deposition of fibrin in which blood fills the defect in the tissue and clots to form a fibrin-rich haematoma. Blood flow slows in the capillaries and polymorphonuclear leukocytes begin to adhere to the capillary walls, which become more permeable, allowing the cells to permeate into the fibrin plug. Hesp et al (1985) studying the healing of inverted ileal and colonic anastomoses in rabbits showed that granulocytes were present in both types of tissue within 3 hours of surgery and reached maximum numbers between 12 and 24 hours. Monocytes and fibroblasts both began to appear at 24 hours and were still in the tissues after 3 to 7 days. The surface columnar epithelial cells did not migrate but mitosis in the glandular crypts provided cells for the restoration of epithelial continuity. In these early stages, before new collagen is laid down, the integrity of the wound depends on the support provided by the sutures and the fibrinous nature of the cellular infiltrate and clot that fills the gap between the cut edges. At this time the intrinsic strength of the anastomosis is at its lowest, collagen has been degraded and new collagen has not yet been laid down in a mature form and it is critical in this period to have a leak-proof repair (Foster and Leaper 1984). Fortunately for bowel anastomosis this is a very short time as the tensile strength is more rapidly restored than in the skin or the abdominal wall. Usually there is intense inflammation in the invaginated margin of mucosa which undergo considerable degradation within 48 hours of surgery. Polymorphonuclear leukocytes release the contents of their cytoplasmic granules and the mononuclear cells become tissue macrophages which show active phagocytosis of extracellular and necrotic material and which stimulate angiogenesis, migration and proliferation of fibroblasts. New blood vessel buds begin to sprout from submucosal capillaries near the cut edges. Lymphatic channels develop more slowly and oedema results from the increased capillary permeability.
The second phase of healing, proliferative fibrous repair, begins between the third and fifth day. Oedema and inflammation decrease, fibroblasts stimulated to migrate by macrophage products gradually predominate in the fibrin plug and synthesise proteoglycans and new collagen to form the fibrous tissue, initially an oedematous granulation tissue composed of new capillaries, fibroblasts, macrophages, mast cells, inflammatory cells and cellular debris. New vessels become canalised developing into arterioles or venules. Collagen production increases, in the mid period of the second phase, initially only reticulin fibres are demonstrable but these are quickly followed by collagen fibrils that are responsible for the intrinsic mechanical strength of the suture line. Low cuboidal mucosal epithelial proliferation over the surface of the invaginated edge and extensive mitoses of proliferating smooth muscle cells are seen at the end of the second phase. The appearance of the anastomosis at this time varies with the extent of sloughing of the invaginated mucosal margin which is dependent on the suture technique. With interrupted sutures close to the cut edges of the bowel there is little inversion of the margins, less necrosis followed by an orderly restorative process with minimal formation of granulation tissue. Excessive sloughing of the inverted edges results in the production of exuberant granulation tissue and healing by secondary intention. Waninger et al (1992) studied the influence of distance and tension of sutures on the healing of experimental colonic anastomoses in rats and found that a wide distance between the interrupted sutures was followed by inadequate apposition due to prolapse of the mucosa between the sutures. Comparing wide and narrow distance with three different tensions he demonstrated that best results were achieved with a narrow distance between sutures combined with a moderate suture tension.

The second phase gradually merges into the reorganisation and remodelling of the third phase that continues indefinitely (Irvin 1984). Firstly, in the submucosa, there is a decrease in the number of fibroblasts and macrophages and the wound becomes less vascular. Many of the new capillaries become occluded and disappear and the wound becomes progressively less cellular with a few remaining inflammatory cells and round
cells or foreign body giant cells usually around buried suture material. The end result is a dense and relatively avascular collagenous scar (Irvin 1984). From 17 to 21 days a hyperplastic mucosa overlies the previous defect and there is a proliferation of smooth muscle cells and collagen fibres at the suture line and the muscularis mucosa begins to separate from the proliferating muscle mass. From three weeks to one year, remodelling results in a thinning of the bowel wall as gradually the layers re-orientate. However the muscularis mucosa remains incompletely formed at one year frequently still indistinguishable from the muscularis propria so that the submucosa is not clearly demarcated.

1.2.2 Biochemical aspects of wound healing

Several chemical mediators are involved in the vascular inflammatory response in the early phase of wound healing. The vasodilatation that follows injury is caused by the release of histamine by platelets and mast cells. The action of histamine is quite brief and the maintenance of capillary engorgement and increased vascular permeability which follows is mediated by serotonin, bradykinin, kallidin and prostaglandins. Serotonin is released by platelets and mast cells. Bradykinin and kallidin are peptides released from plasma globulins by the enzyme kallikrein. Several prostaglandins are involved in the initial phase. These substances are derived from the tissue and from neutrophilic leukocytes and cause vasodilatation and increase the permeability of capillaries. They may also regulate the duration of wound inflammation (Irvin 1984).

Activation of platelets releases platelet-derived growth factors and chemoattractants for fibroblast and vascular endothelial cells. Fibrinogen polymerisation and fibrin lysis releases chemoattractant fibrinopeptides. Blood and tissue complement factors are released which, in conjunction with fibrinopeptides, attract inflammatory cells, which in turn release their own growth factors and chemoattractants. Local cells proliferate and move towards the source of the signals.
Leukocytes and macrophages accumulate, the former rapidly and the latter more slowly (Hunt 1984).

Cytokines, notably interleukin-1, are released by macrophages and have far-reaching effects on connective tissue formation by encouraging proliferation of fibroblasts and on increasing collagen and fibronectin synthesis. Interleukin-1 acts also to promote connective tissue destruction by increasing collagenase, neutral protease and prostaglandin E\textsubscript{2} production (Dayer 1987, Pasternak et al 1986).

1.3 Colonic anastomotic dehiscence

1.3.1 Clinical studies

The fundamentals of intestinal anastomoses were established by the experimental work of Travers, Lembert and Halsted, but much of the knowledge concerning colonic healing was obtained from clinical studies of patients following intestinal anastomoses. Clinical observations by surgeons at the end of 19th century had shown that anastomosis of the left side of the colon carried a higher risk of leakage and infection than anastomosis in any other segment in the gastrointestinal tract (Goligher 1984). Retrospective analysis has been used to determine the clinical course of a large number of patients so that factors associated with either satisfactory or impaired healing could be identified. With this approach many risk factors for anastomotic dehiscence were established. Irvin and Goligher (1973) observed the highest incidence of anastomotic disruption after low anterior resection of the rectum (25%) and in patients with faecal loading of the bowel, either because of poor bowel preparation or intestinal obstruction (24%). Schrock et al (1973) found a high incidence of dehiscence after massive haemorrhage (31.8%), emergency resections (13.3%) and after anastomosis in the presence of infection (10.5% in elective and 12.5% in emergency resections). Charnock et al (1977) reported a higher incidence of anastomotic dehiscence in patients
having a staged resection than in those having a single operation for diverticular disease which he believed was related to a more severe disease and a technically more demanding procedure in those patients requiring a defunctioning colostomy. Phillips et al (1985) reviewing 713 patients with malignant large bowel obstruction observed a high clinical leak rate (18%) with primary anastomosis in the left colon compared to elective surgery (6%).

Since Dixon (1939) advocated anterior resection many controversial reports about the incidence of dehiscence of the suture-line in anterior resection have appeared in the medical literature, some reporting a low incidence but the majority of surgeons agree that there is a substantial risk (Mayo et al 1951; Goligher 1951, 1958, 1962; Goligher et al 1965, Muir 1958; Cullen and Mayo 1963; Whitaker 1968 ). Goligher et al (1970a) in what is now a classic paper evaluated the occurrence of dehiscence in 73 patients who underwent anterior resection for carcinoma or for benign tumour of the rectum or rectosigmoid and for diverticular disease. They employed not only the usual diagnostic criteria based on the clinical course of the patient, septic complications, digital and sigmoidoscopy examination, but introduced the use of contrast radiology to assess anastomotic integrity during the immediate post-operative period. Radiology revealed suture-line leaks that were not clinically apparent and the overall incidence of leakages recorded were 40% and 69% in high and low resection respectively, with 51% for the entire series. They stressed the high frequency of dehiscence and its adverse influence on the post operative course. A 2.7% mortality was attributed to this particular complication and the mean period in hospital after high anterior resection was increased from 14 days in patients whose anastomoses remained intact to 35 days in those whose anastomoses broke down.

Garnjobst and Hardwick (1970) studied 98 resections of the sigmoid colon for diverticulitis, 48 primary resections, 26 two stage resections and 24 delayed resections and found 10 anastomotic leaks (10.2%). In the patients without protective colostomy.
there was one death (1.02%) and one pelvic abscess. They advocated a proximal
decompression for low anastomosis since routine use of post-operative contrast enema
prior to colostomy closure showed 8 asymptomatic anastomotic defects. They believed
that some of the defects at suture lines were related to accumulation of blood and serum
around an anastomosis which drained through the suture line into the lumen of the
colon. To avoid this they advised draining the pelvis long enough to allow liquefaction
of the clot, leaving the drain in place until the danger of leak has passed. Debas and
Thomson (1972) in a critical review of colectomy in 838 patients reported an 8%
anastomotic dehiscence which was responsible for a third of all the deaths in the series,
mainly following emergency operation. Surprisingly they found a higher mortality in
right sided emergency resections than in resections of the left colon. This was
attributed to the application of a one stage resection in those patients with established
peritonitis at the time of right hemicolectomy whereas left side emergency resections
were managed by staged procedures. Morgenstern et al (1972) reviewing 301 cases,
comprising 178 low anterior resections performed for carcinoma and 123 segmental
sigmoid resections for diverticulitis, documented 70 cases of leakage (23%) with 8
deaths (2.5%). In the 178 cases of low anterior resection there were 45 leaks (25%)
with 4 deaths (2%) and in the 123 segmental sigmoid resections they found 25 leaks
(20%) with 4 deaths (3%). They concluded that colostomy should be considered in
selected cases as all the major leaks that resulted in death occurred almost entirely in
those patients undergoing primary resection without preliminary or concomitant
colostomy. Morgenstern and his colleagues suggested that the selection of patients for
colostomy or staged resections should be based on the clinical factors most frequently
associated with leakage such as advanced age, obesity, malnutrition, coagulopathy,
steroid dependence, uraemia and diabetes or local factors for example the presence of
active infection or inflammation (abscess, fistula, peritonitis), gross contamination by
faecal spillage during resection and technical difficulty in performing the anastomosis.
A further factor that may have contributed to dehiscence in those patients undergoing
primary resection was the finding of foci of acute inflammation, submucosal oedema,
muscular hypertrophy and even micro-abscess formation at the resection margin. These findings were present not only in diverticulitis but also in the vicinity of a necrosing invasive carcinoma.

Schrock (1973) in a comprehensive review of 1703 patients with colonic anastomosis over a twenty year period found 4.5% had clinical evidence of suture line disruption. There was a 33% mortality associated with dehiscence, compared to 2.6% when the anastomosis was intact. The incidence of leakage in left colorectal anastomoses was 9.2% and in low rectal anastomoses 10.4%; this was attributed to poor blood supply and exclusion from the peritoneal cavity. Even in anastomoses carried out in ideal circumstances the incidence of low anastomotic leakage was still 10% compared to 1.7% in other colonic anastomosis. Factors which contributed to an increased risk of dehiscence were old age, anaemia, radiation therapy, carcinoma at the margins, infection, intra-operative hypotension, operations lasting more than 5 hours and massive haemorrhage, the latter was associated with a particularly high rate (31.8%) of anastomotic dehiscence. Elective resection of the left colon for diverticular disease resulted in only 0.6% leakage in the absence of infection but if the operation was carried out as an emergency there was disruption in more than 10% of cases irrespective of the associated sepsis.

To elucidate the most important factors in the failure of anastomotic healing Irvin and Goligher (1973) reviewed the case records of 204 patients in Leeds General Infirmary. Clinical evidence of anastomotic dehiscence occurred in 14% of cases and increased considerably in cases where there was faecal loading of the bowel (24%) and after low anterior resection of the rectum (25%). Colostomy did not eliminate the threat of anastomotic disruption. A significant reduction in preoperative plasma proteins was found in patients with anastomotic dehiscence. The incidence of disruption was highest in patients over the age of 60 years but faecal soiling and peritoneal sepsis were not risk
factors, probably mitigated by the use of prophylactic broad-spectrum antibiotics and peritoneal lavage with antibiotic solutions.

Fielding et al (1980) reported a multicentre study of 1466 anastomoses in patients with colorectal cancer. The overall clinical dehiscence rate was 13%; 10.8% in intraperitoneal anastomosis and 18.7% in anterior resection of the rectum and the highest incidence was found in advanced tumours with metastases. The clinical seriousness of anastomotic dehiscence was that the duration of hospital stay was doubled and there was a threefold increase in post-operative mortality when this complication occurred. The most important single factor influencing anastomotic integrity was the surgeon who performed the operation, the range of dehiscence for individual surgeons varying from 5 to 30%.

Recent evaluation has been directed towards the use of stapling devices, usually in low colorectal or coloanal anastomosis. Smith (1981) in a survey of the American Society of Colon and Rectal Surgeons reported that 10% of patients with circular stapled anastomosis had a clinical leak. Fazio (1988) used the circular stapler to perform low and high colorectal anastomosis and compared the leak rates using clinical and radiological criteria. He found of 67 patients having a high colorectal anastomosis rates of dehiscence were 1.5% and 0%, for radiological and clinical leaks respectively; however in 95 patients with a low colorectal anastomosis the rates increased to 8.4% and 5.3%, respectively. The use of staplers has decreased the incidence of low colorectal anastomotic dehiscence but has not abolished it.

Hardy et al (1985) created a biofragmentable anastomosis ring (BAR) that facilitates sutureless, atraumatic anastomosis of the inverted ends of the bowel using a device similar to the Murphy's button, made of a non-reactive synthetic material. The ring passes completely out of the body approximately 18 days after surgery, when mechanical support is no longer needed. Bubrick et al (1991) using this technique in a
randomised multicentre trial of 782 patients reported 3% anastomotic leak rate in sutured, 4% in stapled and 3% in BAR anastomosis however low colorectal or emergency anastomosis were not investigated.

As emphasised by Irvin and Goligher (1973) faecal loading is an important cause of leakage in colonic anastomosis and is a contributing factor in emergency colonic surgery. To obviate this problem surgeons since the end of the last century have used a three staged procedure, a defunctioning colostomy first, followed by bowel resection in the second stage and colostomy closure in the final stage (Devine 1931). In most instances the diseased segment of the colon was removed at the initial operation followed by delayed restoration of the bowel continuity. This two stage procedure is preferred because primary resection eliminates continued contamination of the peritoneal cavity when treating an inflammatory disorder such as acute diverticulitis and achieves more timely removal of the tumour when dealing with a neoplastic process. The efficacy of these diverting operations is questionable because they still carry a certain morbidity and mortality. A variety of technical modifications have been proposed to address some of the potential hazards associated with resection and primary anastomosis of the distal colon in emergency operations when preparation of the colon is not possible. Muir (1968) described a technique of intraoperative lavage of the unprepared colon to facilitate primary anastomosis and a recent report from Murray (1991) showed no anastomotic leakage in 31 patients after emergency left colectomies in the Lahey Clinic with intraoperative lavage of the unprepared bowel followed by primary anastomosis. Ravo and Ger (1984) introduced a technique for protecting colonic anastomoses using an intracolonic bypass tube and Rosati et al (1992) reported only one anastomotic leak using this technique in 29 emergency colonic resections.

Young and Wheeler (1983) explored the effect of a proteolytic enzyme inhibitor, aprotinin, which had been shown to inhibit collagenase in vitro (Latner et al 1973), on anastomotic healing in a prospective randomised trial. The overall
radiological leak rate in 100 patients was 22% for the aprotinin group and 35% for the placebo group, and the overall clinical leak rate was 8% and 15% respectively. They concluded that this reduction in anastomotic failure rate could be of clinical relevance and deserved further studies.

Sheridan et al (1989) in a double-blind, multicentre, prospective trial have also explored the potential value of aprotinin in 216 patients. There was a marked reduction in the overall anastomotic leak rate with the use of aprotinin, in patients undergoing anterior resection who received aprotinin, when compared to those receiving placebo, but this did not reach statistical significance. However in the patients undergoing left hemicolecetomy or sigmoid colectomy higher leakage rates, both clinical and radiological, were higher in patients treated with aprotinin. They believed that although aprotinin could not be recommended for general application in colonic surgery, its use might be of value in improving anastomotic healing in patients undergoing anterior resection.

These were disappointing attempts to manipulate the enzymatic system to improve colonic healing and reflects on a simplistic understanding of a far reaching complex mechanism.

1.3.2 Experimental studies

Although prospective and retrospective clinical studies have identified the common causes of colonic dehiscense and the associated risk factors they have not accounted for the underlying biological and biochemical processes that may be involved in anastomotic breakdown. Any study on the mechanism of colonic healing or its failure in a clinical setting is logistically and ethically not feasible, and researchers have therefore relied on different animal models. Various factors have been investigated related to, surgical technique, suture materials, as well as the effect of
external factors, like anti-inflammatory agents, infection, bowel preparation, nutrition and blood transfusions.

As early as 1812 experimental work carried out by Travers in horses demonstrated the importance of sealing the anastomosis with the serosa to avoid leakage. The first systematic research of colonic anastomosis was by Halsted in dogs who demonstrated that the strength of the bowel wall is reliant on the submucosal connective tissue (Halsted 1887). As the strength of this submucosal tissue depends upon the quantity and quality of the collagen it contains (Hawley et al 1970b), further studies were driven in this direction. Mechanical strength (the resistance of the anastomosis to rupture) and collagen concentration were taken as the main parameters for studying collagen.

Mechanical strength was measured by the longitudinal breaking strength or the tension at which the anastomosis disrupts when the two ends of the anastomotic segment are pulled apart or by determining the resistance of the anastomosis to intraluminal pressure. The pressure at which leakage or rupture occurs is called the bursting strength or bursting pressure.

Anastomotic strength was also assessed by the collagen content using hydroxyproline measurements, an amino acid unique to collagen. Since the healing of the anastomosis is dependent on the balance of collagenolysis, collagen synthesis and collagen maturation, not only is the absolute amount of collagen present important but also the its and quality of cross linking. Therefore the character of anastomotic healing may be better defined by the relative amounts of immature, or poorly crosslinked collagen and mature, or highly crosslinked collagen present (Hendricks et al 1985). Evaluation of the rate of synthesis and deposition of collagen is by radiolabeled proline that forms hydroxyproline by specific hydroxylation hence permitting measurement of newly synthesised collagen.
Other methods used to assess the quality of an anastomosis in experimental situations were local blood flow at the anastomosis (Leaper 1983), anastomotic integrity with contrast radiography (Bubrick 1981) and histological evaluation (Herrmann et al 1964, Daly et al 1972, Trimpi et al 1977).

Herrmann et al (1964) were one of the first groups to use bursting pressure and breaking strength to study colonic anastomosis in rats comparing the data with the morphological picture seen on histology. Their results showed a reduction in these parameters in the first 4 days with a rise above normal after 5 days. This coincided with the first phase of inflammation, however no explanation was made. Cronin et al (1968) studied distal colonic anastomosis in rats using bursting strength and collagen concentration and reached two conclusions. Firstly, during the first 3 days of healing the amount of collagen in the wound margins decreased by 40 per cent, which correlated with a decrease in bursting strength. Secondly, a similar loss of collagen occurred in the uninjured intestine for at least 2.5cm above the anastomosis. At 10 days the collagen concentration was only slightly below normal while the bursting strength was well above normal so that rupture no longer took place at the anastomosis but in the intact intestine. They concluded that large amounts of pre-existing collagen were destroyed and replaced by newly synthesised collagen, so that in effect a tube of new connective tissue was formed in the submucosal layer (Cronin et al 1968). They confirmed these findings in the same year by another study using the specific activity of tritium labelled hydroxyproline as an estimate of collagen content in the healing colon which showed that once the colon was wounded, collagen was destroyed and then replaced along a variable length of its wall. They also demonstrated that the synthesis of new collagen began as early as the second day. Their results suggest that for at least the first 5 days of healing of a colonic anastomosis, destruction of old and synthesis of new collagen occur simultaneously and from the biochemical point of view it is an error to regard this period as a lag phase (Cronin et al 1968).
These changes in collagen content correlating with a decrease and subsequent rise in the bursting pressure of the anastomosis suggests that the mechanical strength of the wall is dependant on its collagen content.

Similar conclusions were reached by Hawley et al (1970b) working in rabbits in the same laboratory. They found a 40 per cent decrease in the bursting pressure on the third post-operative day and by the seventh day the bursting wall pressure had risen to a mean value similar to that of the unoperated colon. The fall in the bursting strength on the third operative day was associated with a fall in the hydroxyproline content of the colon of 27 per cent. By the seventh post-operative day the total collagen content was restored to a mean value similar to the normal colon.

Hawley (1970b) studied the collagenolytic activity of viable explants of colon and other segments of the gastrointestinal tract by measuring the lysis of collagen. The explants were placed in contact with a collagen gel, incubated for 72 hours and the area of lysis determined. He demonstrated that the caecum and the small intestine produce less lysis than the colon and most explants from the stomach failed to produce any. Following colonic anastomosis the collagenolytic activity was enhanced not only in the colon but also in all the other segments of the gastrointestinal tract, including the stomach. These variations in activity in the gastrointestinal tract appeared to correlate with the risk of anastomotic dehiscence at different sites in normal clinical practice, the incidence of anastomotic dehiscence being higher in the distal colon than in the proximal one or the small bowel.

Dunphy (1970) in a review of his laboratory's experimental results concluded that up to 40 per cent of normal collagen is lost in the first four to six days. Simultaneously new collagen is being synthesised, but this is insufficient to bring about an increase in measurable bursting strength of the bowel until the fifth or sixth day, when there is very rapid rise in both collagen content and bursting strength. After ten to twelve days the
normal colon bursts before the anastomotic site partly because of the decreased distensibility of the suture line.

Irvin and Hunt (1974a) studied colonic anastomosis in rats measuring the bursting pressure, total collagen concentration and collagen metabolism with tritiated proline. They confirmed the findings of Cronin and Hawley demonstrating lysis of mature collagen in the early phase of colonic healing and active collagen synthesis in the first few days following anastomosis, however the change in colonic collagen was of smaller magnitude probably because they used a different technique, measuring collagen content in anastomosis dried to constant weight in an oven. The measurements of bursting pressure also showed a different picture as they found that the early phase of healing was not characterised by a loss of tensile strength and the anastomoses on the third postoperative day had gained a significant degree of tensile strength compared to measurements made on the day of the anastomoses. These differences in the results may be due to the measurements being made of total collagen, and tensile strength and bursting pressure being compared not to the normal colon, as were Cronin’s, but with the measurements made on the day of the anastomosis.

They also investigated the effect of trauma on colonic healing (Irvin and Hunt 1974b) as trauma had been shown to have an adverse effect on wound healing (Zederfeldt 1980). Working on rats they studied the influence of intra-abdominal trauma induced by incising the peritoneum of the posterior abdominal wall and taking a biopsy from the psoas muscle, and extra-abdominal trauma created by fracturing the femur, on colonic anastomosis. Distant trauma had no significant effect on colonic healing either in the collagen content or in tensile strength. However, with intra-abdominal trauma the tensile strength of the anastomoses in these animals was significantly lower than that of controls with a notable incidence of anastomotic disruption.
Van der Stappen et al (1992) measured a higher collagenolytic activity in extracts from first post-operative day anastomotic segments compared to extracts from uninjured intestine of the rat. Anastomosis in the large bowel had higher collagenolytic activity than anastomosis in the small bowel. Analysis, by SDS-polyacrylamide gel electrophoresis, of the reaction products of the degradation of fibrillar type I collagen revealed the presence of many fragments, amongst them ones characteristic of mammalian collagenase activity. This was evidence of enhanced degradation of collagen in the anastomotic area during the first post-operative day and an indirect implication of true mammalian collagenase.

Infection is known to compromise wound healing and has been implicated by several authors in the pathogenesis of colonic anastomotic breakdown. Irvin (1976) investigated colonic collagen metabolism in infected anastomoses in the distal colon of rats using radioactive proline and demonstrated changes in collagen metabolism during the early phase of colonic healing. There was a significant reduction on the third post-operative day in the amount of new collagen in infected anastomoses compared to control anastomoses without infection. This could be related to either impaired synthesis or increased lysis of collagen. Either could account for the reduction in mature collagen observed on the seventh post-operative day in infected anastomoses. Le Veen et al (1976) looked at the effect of prophylactic antibiotics on the healing of bowel anastomosis in dogs and found a significantly increased tensile strength seven days after the operation in the animals receiving antibiotics. Histological examination showed healing by second intention in the animals not receiving antibiotics compared to healing by first intention in those receiving antibiotics. Irvin and Hunt (1974b) working on traumatised rats with infection found a significantly decreased collagen content in colons in which the anastomoses had disrupted. Yamakawa et al (1971) simulated diverticulitis in dog colons and demonstrated a markedly diminished hydroxyproline content in colonic anastomoses in these animals. Cohen et al (1985) demonstrated that enteral antibiotic administration dramatically improved the healing of
anastomosis of ischaemic colon in the rat. Anastomotic healing in unprepared ischaemic rat colon was severely impaired with an 83 per cent dehiscence rate, but this was not observed in the colon prepared with enteral antibiotics. Dunphy (1970), based on experiments by Hawley (1969), stated that bacterial growth on the surface of the anastomosis promoted collagenolytic activity and accelerated breakdown of the anastomosis.

Other factors influencing collagen metabolism were studied by Jiborn et al (1978, 1980a,b) and Jonsson et al (1985) who in a series of experiments in rats demonstrated that collagen concentration decreased in the vicinity of anastomoses by 30 per cent on the first 4 days and this decrease was higher when continuous suture was used or when colonic dilatation occurred proximal to the anastomoses. In these circumstances the collagen concentration was even 45 to 55 per cent lower on the fourth post-operative day. They found a higher collagenolytic activity proximal to the anastomosis than distal to it and also activity was higher in the colon than in the small bowel. After the first 2 to 4 days a gradual restoration of collagen concentration occurred when synthesis compensated for lysis. Tornqvist et al (1988, 1990a, 1990b) in a series of experiments in the rat investigated the effect on collagen turnover of obstructing the colon. An obstruction in the distal colon was found to stimulate collagen synthesis not only at the obstructed site but also proximal to it. This resulted in an increase in collagen content proximal to the obstruction but not at the obstruction site indicating that there must also be an increase in collagen degradation here. Collagen metabolism following an anastomosis either alone or preceded by obstructing the colon and resection of the obstructed segment was also studied. No difference in collagen content was measured between the groups, however in the obstructed group collagen synthesis proximal to the anastomosis, but not at or distal to it, was twice as high implying that collagen lysis must also be occurring at a much greater rate in this region. The major difference between the groups was in the complication rate which was 2 per cent in the anastomosis only group compared to 27 per cent in the obstruction and anastomosis
animals. This study shows that an obstruction does have a profound effect on collagen dynamics with both synthesis and degradation being affected.

However the first direct demonstration of collagenase in the anastomotic site was made by Chowcat et al (1988) using an immunohistochemical method. They localised collagenase and TIMP-1 in the suture line of colonic anastomoses formed in rabbits after resection of a small segment of distal colon. Collagenase and TIMP-1 were confined to a narrow margin at the cut ends of the bowel wall. The enzyme appeared in the mucosa within 12 hours of operation and gradually progressed more deeply through the damaged edges, initiating collagen breakdown in the tissue prior to its infiltration by granulocytes. By 24 hours collagenase secretion was already beginning to wane and TIMP-1 appeared, suggesting tight control of collagenase activity. Three days after operation much less collagenase was localised and TIMP was seen in the serosal sealing layer probably to prevent protease degradation and maintain the anastomotic integrity. After 7 days both enzymes and inhibitor were active only in small areas where dying tissue remained to be destroyed and where remodelling was taking place. The enzyme performed a controlled process of breakdown of unwanted tissue and there was no evidence for excessive collagenase activity in the normal healing of a colonic anastomosis. In another study Chowcat et al (1990) measured colonic secretion of collagenase in tissue culture and further demonstrated that three days after anastomosis the colon secreted more collagenase than explants of one day postoperative tissue. This coincided with a decrease in collagen concentration in the colon wall measured as hydroxyproline.

The data suggest that after colonic anastomosis collagen metabolism is considerably enhanced. In the first 4 days after operation breakdown is higher than synthesis and collagen concentration and total collagen content fall. Between 4 and 7 days there is a rapid accumulation of new collagen and collagen concentration at 7 days is higher than in normal colon. Measurements of bursting pressure and tensile strength
seem to support these changes. Thus collagen is the most important component of the extracellular matrix and the mechanical strength depends on its content in the bowel wall. Increased collagenolysis has been implicated by indirect studies and recent directed measurements have demonstrated the role of collagenase.

Increased knowledge of the biochemistry of collagen degradation and of the components of the extracellular matrix in particular of the enzymes that specifically degrade these components, deserve a note and point to the gaps in our understanding of colonic healing.

1.4 Extracellular matrix and basement membrane

The extracellular matrix (ECM) is the structurally stable material that lies under epithelia and surrounds connective tissue cells. The ECM, however, is not an inert supporting material, it interacts with the matrix cells and influences cellular proliferation, shape, polarity and differentiation (Hay 1991). Four major classes of macromolecules form the basic extracellular matrix, collagen, proteoglycans, elastin and glycoproteins (e.g. fibronectin, laminin and chondronectin).

Another important structural component of tissues is the basement membrane (BM) which forms an interface between connective tissue, epithelial cells and muscles. Basement membrane consists of a central compact sheet of collagen and other glycoproteins forming the lamina densa, that is separated from the cells by a less electron-dense zone, the lamina rara externa and from the underlying connective tissue by another less electron-dense zone, the lamina rara interna. Both the laminae rarae externa and interna contain a layer of proteoglycan granules connected by small filaments to the cell, on the one side, and to underlying collagen fibrils, on the other (Hay 1991). The BM owes its strength to the presence of collagen, mainly type IV, which forms a network with heparan sulphate-containing proteoglycans and the
glycoproteins, laminin and fibronectin. The BM is thought to serve as a scaffold for
organising and anchoring epithelia. In spite of having a composition similar to the
ECM and "connecting" epithelia and muscle to the ECM, the BM is not a component of
the connective tissue (Hay 1981).

Study of the ECM must consider its synthesis, composition, organisation and
degradation. The most important component of the matrix is collagen.

1.4.1 Collagen

Collagen is the most abundant protein in animals constituting more than half of
the total protein in adult organisms and is the principal component of the ECM
(Linsenmayer 1991). The collagen molecule gives support and tensile strength to skin,
bone, tendon and cornea of vertebrates and is the main structural component of many
tissues, forming 72% of the dry weight of skin and 86% of Achilles tendon (Uitto et al
1986); it is mineralised to produce a rigid skeleton in bone. In the intestine, where it
constitute 15% of the dry weight (Gottrup 1981), it forms a flexible supporting layer
which allows expansion of the lumen and movement of the contents without loss of the
integrity or the elasticity of the wall (Jackson 1980).

Collagen molecules are formed of α-chains of which 24 different ones are
known resulting in at least 15 types of collagen. The types found in a tissue are related
to its structure and function. Collagen is not only an important structural component
but can also promote cell attachment and differentiation, is a chemoattractant for both
macrophages and fibroblasts, an antigen in immunological processes (Linsenmayer
1991). Defective collagen results in pathological conditions, for example, osteogenesis
imperfecta, Ehlers-Danlos syndrome and various types of chondrodysplasias (Montes
and Junqueira 1982).
1.4.1. i Molecular structure

*Triple helical region* - Collagen molecules are rod-like structures usually 3000 Å long and 15 Å in diameter (Gross 1981). In the fibril forming collagens (types I, II, III, V and XI) more than 95% of the molecule is composed of a single triple helical domain that gives collagen its characteristic shape and properties. This region consists of three separate α-chains, each of which contains approximately 1000 amino acids, twisted in the form of left-handed helix. These three chains are then wrapped around one another to produce the triple helical structure of the molecule (Linsenmayer 1991). The triple helical conformation is such that the peptide bonds linking adjacent amino acids of the chains are buried within the interior of the molecule, making it highly resistant to attack by general proteases such as pepsin. The only enzymes able to efficiently degrade the helical portion of native collagen molecules are the collagenases (Gross et al 1980).

The helical nature of the molecule is possible because of the presence of glycine at every third residue in the chain, since only the small side group of glycine will fit in the centre of the helix giving approximately 333 glycine residues per chain. The chains consist of repeated sequences of Gly-X-Y, where X and Y are frequently proline and hydroxyproline, these two amino acids accounting for about 20% of the total amino acid content, each occurring at about 100 sites per chain. Hydroxyproline and hydroxylysine (the enzymatically modified form of lysine), are both found almost exclusively in collagen and participate in intra- and inter-molecular cross-links stabilising the helix and fusing the molecules into fibrils (Burgeson 1982, Pinnell 1983, Forrest 1983, Uitto et al 1986). The length of the helical section determines the nature of fibril formation in the various collagen types. Combinations of different types of collagen perform a wide range of functions in a variety of tissues and sites. Fibrils collect into fibres and bundles of parallel fibres form the rope-like collagen of tendon, known for its tensile strength. In the cornea the fibres are orientated at right angles to
allow transparency and, in skin, a more random pattern gives flexibility (Pinnell 1983). The collagen fibres of the submucosa in the small intestine form a complex mesh, are straight, lie parallel to the serosal surface and are oriented in different directions with a criss-cross pattern that changes with the movements of the intestine, the angle formed by the two arrays of collagen fibrils with the length of the gut increases to 60°-65° when the radial distension predominates, while it is reduced to about 30° when the longitudinal distension predominates. This arrangement gives strength to the wall, allowing it to be stretched by a considerable amount both along and across the longitudinal axes of the intestine (Gabella 1987).

Non-triple helical region - In addition to the triple helical region, collagen molecules in the ECM also have short non-helical extension of approximately 20 peptides at the NH$_2$- and COOH-terminal ends of each component alpha chain. These terminal extension peptides play important functional roles, for they represent the sites where hydroxylysine-derived intramolecular and intermolecular cross-links are formed when molecules are arranged in fibrils. As the cross-linking containing extension peptides are not in the triple-helical conformation they are susceptible to proteolytic degradation by general proteases under conditions in which the triple-helical body of the molecule is left intact (Linsenmayer 1991).

1.4.1. ii Genetic types of collagen

At least fifteen collagen types with twenty four α-chains have been purified and described (Anderson 1992) (Table 1.1). For each collagen type a Roman numeral has been assigned reflecting the chronological order in which it was discovered (Bornstein and Sage 1980). They are also identified by their α-chains composition. By convention the different chains of a molecule are designated α1, α2, α3 with the collagen type to which they belong designated by the Roman numeral, e.g. the α1 chain of type I collagen is α1(I) and its α2 chain is α2(I). The complete designation of a
## Table 1.1. Collagens

<table>
<thead>
<tr>
<th>Class</th>
<th>Type</th>
<th>Chain composition (most common form)</th>
<th>Common distributions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrillar</td>
<td>I</td>
<td>[a1(I)]2 [a2(I)]</td>
<td>Fibrous stromal matrices</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>[a1(II)]3</td>
<td>Cartilage, vitreous, primary corneal stroma, notochord</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>[a1(III)]3</td>
<td>With type I in heterotypic fibrils</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>[a1(5)]2 a2(V)</td>
<td>With type 1 in heterotypic fibrils; thin fibrils</td>
</tr>
<tr>
<td></td>
<td>XI</td>
<td>a1(XI) a2(XI) a3(XI)</td>
<td>with type II in heterotypic fibrils</td>
</tr>
<tr>
<td>Fibril associated</td>
<td>IX</td>
<td>a1(IX) a2(IX) a3(IX)</td>
<td>Surface of type II collagen fibrils in cartilage; primary corneal stroma, notochord</td>
</tr>
<tr>
<td>Network forming</td>
<td>IV</td>
<td>[a1(IV)]2 a2(IV)</td>
<td>Basement membranes</td>
</tr>
<tr>
<td>Filamentous</td>
<td>VI</td>
<td>a1(VI) a2(VI) a3(VI)</td>
<td>100-nm beaded filaments of stromal matrices</td>
</tr>
<tr>
<td>Short chain</td>
<td>VIII</td>
<td>?</td>
<td>Descemet's membrane, subendothelial matrices</td>
</tr>
<tr>
<td>Long chain</td>
<td>X</td>
<td></td>
<td>Hypertrophic cartilage</td>
</tr>
<tr>
<td></td>
<td>VII</td>
<td>[a1(X)]3</td>
<td>Anchoring filaments</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[a1(VII)]3</td>
<td></td>
</tr>
</tbody>
</table>
molecule also includes the ratio in which the chains occur. Thus, the type II collagen molecule with its single type of chain is designated \([\alpha_1(II)]_3\) and the type I collagen molecule with its two different chains is \([\alpha_1(I)]_2 \alpha_2(I)\). Collagens types are classified in six classes, determined either by the supramolecular forms in which they occur within extracellular matrices or by their molecular size as compared to the prototypical type I collagen, as seen in Table I.

The fibrillar collagens, types I, III and V, will be described in more detail because they are the most important ones in the study of wound healing. They are the major connective tissue collagens and are the proteins that provide the strength for the submucosal layer of the bowel. Type IV, the main component of basement membranes, will be also described.

1.4.1. iii The fibrillar collagens

The fibrils of many tissues are heterotypic structures, composed of different combinations of fibril-forming collagens co-assembled with one another (Birk et al 1988, Linsenmayer 1991). The structural similarities of the fibrillar collagen molecules allow them to combine and this co-assembly of various collagens can determine certain properties of fibrils in different tissues, for example, fibril diameter (Birk et al 1990).

The fibrillar interstitial collagens types I, II and III, are the major connective tissue collagens. They have a basic molecular weight of 300 000 (Uitto et al 1986), with each polypeptide chain being just over 1000 amino acids long (Murphy and Reynolds 1985a); the triple helices aggregate to form fibrils and bundles of fibrils make up the collagen fibres. Type I is the prototype of fibrillar collagen and the most abundant, made by fibroblasts, osteoblasts and odontoblasts, and found particularly in situations were tension occurs. Its main function is structural and it is found in skin, bone, dentine, tendon, fascia, organ capsule, fibrous cartilage, sclera (Montes and
Junqueira 1982) and is also a major component of intestinal wall, chiefly in the submucosa (Orberg et al 1982, Fogdestam and Gottrup 1980). Type I molecules are 300 nm long and are composed of two $\alpha_1$(I) chains and one $\alpha_2$(I) chain. Type II synthesised by chondroblasts is the major collagen in cartilage, having an intense interaction with proteoglycans, which is responsible for the resistance of cartilage to the intermittent action of pressure (Montes and Junqueira 1982). Type III has a tissue distribution similar to type I, except for bone, tendon and cornea where there are a very small amount. The use of specific antibodies to collagen suggests that type III is the principal component of the small argyrophilic collagen fibres that characterise reticular connective tissue, classically known "reticular fibres" of histologists (Linsenmayer 1991, Montes and Junqueira 1982). This type of collagen is usually found co-assembled with collagen type I as in uterus, skin, arteries, lung, spleen, liver, kidney. It exists in many organs and is mainly, but not exclusively, related to smooth muscle cells. In intestine type III collagen is present in muscular layers and lamina propria. Collagen type III is produced by smooth muscle cells, fibroblasts, reticular cells of haemopoietic organs, liver cells and Schwann cells of the peripheral nervous system and it is always present in tissues and organs that need a mobile structural scaffolding, such as arteries, muscular layers of the intestine and uterus (Montes and Junqueira 1982).

The predominant molecular species of type V found in most tissues is the heterotrimer $[\alpha_1(V)]_2\alpha_2(V)$ but it has been reported that molecules of composition $\alpha_1(V)\alpha_2(V)\alpha_3(V)$ may occur (Linsenmayer 1991). Collagen type V and type XI represent quantitatively minor fibrillar components that co-polymerise with the major types. They are buried in the fibril and immunologically masked (Birk et al 1988, Mendier et al 1989) so that the antibodies against both types V and XI fail to react unless the fibrils have been disrupted by either physical or chemical methods (Linsenmayer 1991). Collagen types I, III and V are usually found in the same tissues except in bone where type III is missing. Collagen types II and XI are found in
cartilage (Van der Rest and Mayne 1988). Collagen type V may be involved in regulating fibril diameter, the greater the percentage of type V collagen in a heterotypic fibril, the smaller the diameter of fibril formed (Birk et al 1990). Type XI is the cartilage equivalent of type V.

Collagen type IV is synthesised in epithelial and endothelial cells and found in basement membranes. It has two main functions: the first as a structural support for epithelia and secondly for its filtering capacity in the renal glomerulus (Montes and Junqueira 1982). The helical region is much interrupted, so fibril formation does not take place and the molecule contains domains including collagenase sensitive triple-helical ones, pepsin sensitive non-helical ones and ones that are resistant to both pepsin and collagenase (Linsenmayer 1991).

The fibril associated collagens type IX and XII can effect interactions between fibrils and between fibrils and other components of extracellular matrices (Anderson 1992). Type VI collagen has an anchoring function forming microfibrils linking major fibres (Keene et al 1988). Type VII forms anchoring fibrils to basement membranes (Lunstrum et al 1987); Type X is associated with calcifying cartilage (Thomas et al 1990) and collagens type VIII, XIII, XIV and XV are of unknown function (Anderson 1992).

1.4.1.iv Collagen biosynthesis

Because of the complicated structure of the collagen genes, the processing of the primary RNA transcripts to form mRNA is very complex. The best understood is the synthesis of fibrillar collagens which will be used as a model for collagen synthesis. Two phases will be considered, firstly, a series of intracellular steps leads to the synthesis and assembly of procollagen molecules and secondly, the procollagen is
transformed extracellularly to tropocollagen and incorporated into a stable cross-linked fibrillar structure (Montes and Junqueira 1982, Laurent 1987).

Collagen genes contain coding regions (exons) interrupted by non-coding intervening sequences (introns). The initial RNA transcripts of the genes are copies of both exons and introns. During processing of the RNA, splicing enzymes remove the intron sequences, producing mature mRNA molecules (Olsen 1991). The α-chain precursor (pre-pro-α) is synthesised on the rough endoplasmic reticulum and after several modifications by membrane bound enzymes (hydroxylation, glycosylation) and other enzymatic steps in the Golgi apparatus the pro-α-chains are completed and pass down the cisternae of the endoplasmic reticulum where three chains assemble to form a triple helix soluble procollagen (Olsen 1991).

Procollagen passes through the Golgi complex, is packaged for secretion and leaves the cell by exocytosis. In procollagen each chain has large additional extension-peptides, called propeptides, at both NH₂ and COOH terminals (Tanzer et al 1974, 1976, Byers et al 1975). Prior to fibrillogenesis, at least two type-specific proteinases act: N- and C-terminal proteinases cleave their respective propeptides (Anderson 1992), leaving the long triple helix region with short non-helical ends characteristic of the collagen molecule, known at this stage as tropocollagen. Spontaneous self assembling into fibrils occurs due to the formation of a series of covalent bonds, creating the cross-links which are responsible for the stability and strength of collagen (Montes and Junqueira 1982, Laurent 1987, Anderson 1992).

Control of protein synthesis can be exerted through control of mRNA levels by regulation of gene transcription and RNA maturation or the stability of mRNA. In the case of collagen there is generally good correlation between the rate of collagen synthesis and the level of mRNA in the cell. Several types of data collectively indicate that the regulation of fibrillar collagen synthesis by cells in culture or in developing
organisms occurs primarily by regulation of the mRNA levels rather than by control of mRNA translation (Olsen 1991).

1.4.1.v Collagen degradation

Collagen has a very important structural role as the major organic constituent of the connective tissue matrix of animals. Increased deposition of collagen in tissues is dependent on the relationship between rates of synthesis and degradation. Collagens are complex fibrous protein that function as the major structural proteins of the connective tissue matrix of animals because of their characteristic rigidity and resistance to proteolytic attack. The traditional view that collagen is metabolically inert is no longer tenable. The turnover of collagen is seen as very slow in adult animals, however it can occur in normal tissues at quite rapid rates in special cases. In skin of adult rats, the mean rate of collagen turnover lies between 3 and 5%/day, but rates of 10%/day may occur in some tissues such as lung or periodontal ligament (Laurent 1987). A continuous slow process of turnover does take place with a half-life between 50 and 300 days (Murphy and Reynolds 1985a) but can be significantly increased in special circumstances, such as growth, involution of the post partum uterus and wound healing. Excessive degradation can occur in some diseases, for example, arthritis a condition that has provided much of the information about the mechanisms of collagen breakdown (Sellers and Murphy 1981).

Complete collagen degradation is usually the result of the synergistic action of several matrix metalloproteinases. The process is best illustrated by the degradation of collagen type I by interstitial collagenase, which degrades collagen type I, II, III, VII and X. The triple helix structure of tropocollagen is cleaved by interstitial collagenase at a locus three quarters of the way between the amino- and carboxy- termini of the molecule. This occurs at a Gly-Ile bond in the α1(I) chains and at a Gly-leu bond in the α2(I) chain. The resultant fragments are named 3/4(TCA) and 1/4(TCB) (Harris and
Krane 1974) and are unstable and spontaneously untangle to allow further degradation by gelatinase and other proteases (Murphy and Reynolds 1985a).

Intracellular degradation of collagen may be the means by which defective molecules are destroyed before they are secreted (Bienkowski 1984). Phagocytosis of damaged fibrils and fragments produced by the extracellular pathways is also known to occur. Once taken up, the fragments can be digested by lysosomal cathepsins which act at acid pH (Murphy and Reynolds 1985a).

Although collagenase is seen as the key enzyme in collagen degradation under normal circumstances a second extracellular pathway can occur when the pericellular environment becomes acidic. Serine and cysteine proteases, secreted by polymorphonuclear leukocytes and other inflammatory cells, are able to degrade collagen directly at acid pH. Elastase, a serine protease, can attack the non-helical terminal regions breaking down intermolecular cross-links to produce free α chains which can be taken up by phagocytes. Cathepsins B, L and N are cysteine proteinases which may also act on the cross-links in acidic milieu (Sellers and Murphy 1981, Murphy and Reynolds 1985a).

1.4.2 Proteoglycans

Proteoglycan molecules form a highly hydrated gel-like ground substance in which fibrous proteins are embedded. They are secreted principally by fibroblasts or by cells of the fibroblast family, for example chondroblasts in cartilage and osteoblasts in bone. They are hybrid molecules consisting of a protein core to which are attached long chains of highly sulphated repeating disaccharides called glycosaminoglycans (Rawe et al 1992). These macromolecules are found inside cells, on the cell surface and in the extracellular matrix of all mammalian tissues and are especially prominent in connective tissues. In cartilage, for example, the proteoglycan represent some 5-10% of
the tissue wet weight (Heinegard and Oldberg 1989). As they form porous hydrated gels, however, the proteoglycans fill most of the extracellular space, providing mechanical support to tissues while still allowing the rapid diffusion of water-soluble molecules and the migration of cells.

Proteoglycans differ markedly in protein content, molecular size and the number and types of glycosaminoglycan chains per molecule. Although there is always an underlying repeating pattern of disaccharides the length and composition of the glycosaminoglycan chains can vary greatly, as can the spatial arrangements of hydroxyl, sulphate and carboxyl side groups along the chains. Due to the variability of the molecule it is very difficult to classify proteoglycans only by their structure, so classifications are usually based on localisation and function.

Wight and Meeham (1987) described them as:

<table>
<thead>
<tr>
<th>type</th>
<th>examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular</td>
<td></td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td></td>
</tr>
<tr>
<td>Large interstitial proteoglycans</td>
<td>aggrecan, versican</td>
</tr>
<tr>
<td>Small interstitial proteoglycans</td>
<td>biglycan, decorin</td>
</tr>
<tr>
<td>Basement membrane proteoglycans</td>
<td>perlecan</td>
</tr>
<tr>
<td>Cell surface proteoglycans</td>
<td></td>
</tr>
<tr>
<td>Intracellular</td>
<td></td>
</tr>
<tr>
<td>Storage granule proteoglycans:</td>
<td>serglycin</td>
</tr>
</tbody>
</table>

1.4.2.i Extracellular

*Hyaluronic acid*

Hyaluronic acid is a relatively simple molecule consisting of regular repeating sequence of non-sulphated disaccharide units. Because of its simplicity, hyaluronic acid is thought to represent the earliest evolutionary form of glycosaminoglycan but it is not a typical proteoglycan as it contains no sulphated sugars and is not covalently linked to protein. It is found in variable amounts in all tissues and fluids in adult
animals and is especially abundant in early stage of embryonic development. There is increasing evidence that hyaluronic acid has a special function in tissues where cell migration is important such as during development and wound healing. Hyaluronic acid is produced in large amounts during periods of cell migration but once migration has ended, the excess acid is degraded by hyaluronidase. This sequence of events has been demonstrated in a wide variety of tissues suggesting that increased local production of hyaluronic acid, which attracts water and thereby swells the matrix, may be a general strategy for facilitating cell migration during morphogenesis and repair. (Alberts et al 1989).

*Large interstitial proteoglycan*

The major proteoglycan isolated from cartilage (aggrecan) is the most widely studied member of this group and as this shares some structural features with other large proteoglycans it will be discussed as a prototype for these molecule. It is a high molecular mass aggregating species and is composed of a central filament of hyaluronan to which numerous proteoglycan monomers are attached (Hardingham et al 1976). The interaction of the proteoglycan monomer with hyaluronan is stabilised by a link protein (Hardingham 1979). The monomer consists of a protein core containing three globular domains (Morgelin et al 1988) G1, G2 and G3 to which chondroitin sulphate and keratan sulphate glycosaminoglycan chains are attached.

Major constituents of cartilage are the fibril-forming collagen type II and aggrecan. Collagen creates strength and the proteoglycans are responsible for the compressive stiffness of the tissue (Kempson et al 1976). Collagen fibres reinforce the mechanically weak proteoglycan gel and the orientation of its fibres can be identified by the direction in which the tissue withstands tensile forces. The proteoglycan gel has a high fixed-charge density, which leads to a large osmotic swelling pressure (Heinegård and Oldberg 1989). It is the balance between the pressure created by the
proteoglycans and the tension in the collagen network that results in the compressive
properties characteristic of the tissue (Hardingham and Bayliss 1990).

Soft tissues appear to contain two types of large proteoglycans with structural
similarity to the one described above. One, versican has been isolated from human
fibroblasts has homology to aggrecan but lacks a G2 domain (Zimmerman and
Ruoslahti 1989). The other which does contain the G2 domain has been isolated from
sclera and tendon and has a dermatan sulphate side chains (Cöster 1991).

Small interstitial proteoglycans

Three low molecular weight proteoglycans, decorin, biglycan and fibromodulin,
are members of a lecitin rich family of proteins and are distributed throughout most
connective tissues. They predominate in fibrous connective tissue forming as much as
95% of the interstitial proteoglycans (Heinegard and Oldberg 1989). Decorin and
biglycan carry one or two chondroitin/dermatan sulphate chains respectively and
fibromodulin bears keratan sulphate chains. Decorin and fibromodulin are likely to
have important roles in organising the extracellular matrix, as each binds to collagen
types I and II in in vitro assays, resulting in delayed fibril formation and the formation
of thinner fibres. Biglycan does not bind to collagen and its functional property is not
apparent. It accumulates at the cell surface and in pericellular environments
(Hardingham and Fosang 1992).

Basement membrane proteoglycans

Heparan sulphate proteoglycan and chondroitin sulphate proteoglycan are
integral components of the extracellular matrix of basement membranes. They were
purified from a murine basement membrane-producing tumour Engelbreth-Holm-
Swarm sarcoma (Hassel et al 1980, 1985). The heparan sulphate proteoglycan can
interact with itself and with other basement membrane macromolecules including type IV collagen and laminin. A smaller heparan sulphate proteoglycan was isolated from the glomerular basement membrane (Stow et al 1985). These proteoglycans contribute to basement membrane architecture, are essential for maintenance of the selective permeability properties of the glomerular basement membranes (Wight and Meeham 1987), bind protease inhibitors such as antithrombin III and facilitate the attachment of cells to their underlying basement membrane (Clement et al 1989).

1.4.2.ii Cell surface proteoglycans

Some of the proteins that are expressed at the cell surface carry one or more glycosaminoglycan chains, either heparan sulphate or chondroitin sulphate glycosaminoglycans. A growing body of evidence suggests that these molecules are primarily involved in the control of cell-cell and cell-matrix interactions. The structural determinants of these biological effects are localised in both the glycosaminoglycan and the protein moieties of these components. The heparan sulphate proteoglycans occupy a special place amongst these substances. By binding to other cell membrane molecules and matrix components the complex highly charged carbohydrate chains of these proteoglycans seem to function by affixing cells and tissues together via "footpad" adhesion sites on the plasma membrane with attachment to collagens I, III and V, laminin, fibronectin and platelet factor IV (Anderson 1992).

Proteoglycan containing cell surface proteins are mainly expressed by fibroblasts but are found on other cell types, including epithelial cells. Proteoglycans identified as having cell surface actions are syndecan, thrombomodulin, betaglycan, CD44 (Hardingham and Fosang 1992), fibroglycan and glypican (David 1991). The matrix- and cell-binding properties of these proteoglycans that have a high affinity to type V collagen, seems to complement the molecular mechanism of cell-cell and cell-matrix adhesion that are based on receptor structures of the integrin and
immunoglobulin super-families. Heparan sulphate proteoglycans play an important role in the extracellular storage and stabilisation of the heparin-binding growth factors that are inducers of cellular differentiation and are involved in embryonic development, angiogenesis and tissue repair (David 1991). They also act in extracellular proteolysis, for example the inactivation of thrombin by anti-thrombin is heparin dependent and also heparin plays a physiological role in inflammation.

1.4.2.iii Intracellular proteoglycans

Storage granule proteoglycan

Most of the mast cells in connective tissue have storage granules which contain a distinct proteoglycan, serglycin. The core protein of the human serglycin has been determined from cDNA clones prepared from a human promyelocytic leukaemic cell line (Avraham et al 1988). The glycosaminoglycan chains are either heparin or an over-sulphated form of chondroitin sulphate. Mast cells produce proteoglycan with a high concentration of heparin that interacts with the cationic proteases, carboxypeptidases, histamines and other molecules which the cell stores for release when participating in a host defence reaction (Wight and Meeham 1987).

1.4.2.iv Biosynthesis and degradation of proteoglycans

The biosynthesis of proteoglycans begins with the synthesis of the protein core in the cisternae of the rough endoplasmic reticulum. Oligosaccharides are added and modified in the Golgi apparatus and glycosaminoglycan chains are built up on the protein core by addition of sugar residues catalysed by a specific glycosyl transferase. Still in the Golgi, sulphate groups and oligosaccharides are attached by transfer of single sugar residues in a process also catalysed by specific transferases. The
proteoglycan is then secreted from the cell, in the case of aggregating proteoglycans as a complex with a link glycoprotein (Anderson 1992).

Since proteoglycans are conjugates of protein and sulphated polysaccharides complete proteoglycan degradation requires the action of proteinases, glycosidases and sulphatases (Murphy and Reynolds 1993). Degradation is initiated by limited extracellular digestion, with proteolysis by neutral metalloproteinases, matrilysin being the most effective, although stromelysin-1 also efficiently degrades at its optimum pH of 5.5. Stromelysin-2, gelatinases and collagenase have a lower activity (Murphy et al 1991). Partially degraded proteoglycans then enter the cell by receptor-mediated endocytosis and intracellular digestion is carried out by lysosomal proteinases (cathepsins), glycosidases and sulphatases (Anderson 1992).

1.4.3. Glycoproteins

Extracellular matrix glycoproteins form interactions with cells and with other matrix components. Those glycoproteins which interact with cells contain a short amino acid sequence recognised by receptors on the cell membrane. Other ECM glycoproteins contain heparin-binding, other related glycosaminoglycans-binding and collagen binding domains. The two most common glycoproteins are fibronectin and laminin.

1.4.3.1 Fibronectin

Fibronectin is the most important ECM glycoprotein being distributed in most connective tissues usually in association with interstitial collagen. Collagen and fibronectin form an integrated fibrillary network in vivo that influences the adhesion, motility, growth and differentiation of cells. Following surgery fibronectin is one of the first components of repair tissue to be deposited.
The basic structure of fibronectin is similar to other glycoproteins but alternative splicing in the cell binding domains gives the molecule its specific characteristics. There are two types of fibronectin, plasma and cellular fibronectin. Plasma fibronectin occurs in blood as a soluble protein at a concentration of 0.3g/litre and is synthesised by hepatocytes. The cellular form is produced by epithelial cells and fibroblasts.

In the extracellular matrix an important role for fibronectin appears to be mediating attachment of cells to collagen. It is able to bind to collagen types I, II, III, IV and V preferring collagen's non-helical region and hence has greater affinity for denatured collagen. Heparin and heparan sulphate enhance the binding of fibronectin to denatured collagen and also stimulate phagocytosis by macrophages of fibronectin mediated uptake of gelatinases particles. In vivo heparan sulphate probably is the molecule involved as heparin only occurs intracellularly.

In wound healing, fibronectin may play a key role providing a scaffold for incoming cells to attach to. This matrix is formed by cross linking to fibrin or fibrinogen through the action of factor IIIa transglutaminase. Immunological studies show fibronectin in colonic anastomoses and skin wounds immediately after surgery distributed along the strands of fibrin. Later in the healing process fibronectin is seen associated with newly synthesised collagen predominantly type III and as the tissue matures fibronectin gradually diminishes. During this remodelling, when collagen turnover is high, fibronectin may play an important role in the removal of collagen by binding to collagen fragments generated by collagenase digestion and promoting their phagocytosis by macrophages and fibroblasts.
1.4.3.ii Laminin

Basement membranes form barriers which in the embryo probably results in the segregation and differentiation of cells into specific tissues. In the adult they form scaffolding to maintain normal tissue form during regeneration and growth and act as molecular filters in capillaries and glomeruli.

One of the principals components of basement membranes is laminin, a large molecule with many functional domains. It is a cross shaped structure formed of three short and one long arm containing three distinct chains. Laminin can bind to matrix components and to cells. It causes secretary cells to become polarised and neural cells to extend axonlike process. Importantly in wound healing laminin can stimulate cell migration and differentiation. Laminin therefore is both a structural and a regulatory molecule.

1.4.4 Elastin

Tissues such as skin, blood vessels, lungs and bowel require elasticity in order to function. A network of elastic fibres in the extracellular matrix of these tissues gives them the ability to recoil after transient stretch. The main component of elastic fibres is elastin, a highly hydrophobic non-glycosylated protein which is rich in proline and glycine, contains little hydroxyproline and no hydroxylsine. After secretion from the cell the elastin molecules, similarly to collagen, form filaments and sheets in the extracellular matrix by cross-linking to generate an extensive network. The polypeptide backbone of elastin remains unfolded as "random coils" and this structure allows the network to stretch and recoil like a rubber band. Collagen fibrils are interwoven with the elastic fibres to limit the extent of stretching and prevent the tissue from tearing (Uitto et al 1991).
Elastic fibres also contain a glycoprotein that is distributed mainly as microfibrils on the elastic fibre surface. Microfibrils are found grouped in small bundles near the plasma membrane and help the cell organise the secreted elastin molecules into fibres and sheets in the extracellular matrix (Uitto et al 1991).

Physiological turnover of elastin appears to be very slow, with a half-life approaching that of the animal (Shapiro et al 1991). Increased elastin destruction does take place in certain pathological conditions as a result of the release of powerful elastases from inflammatory cells or bacteria although these enzymes are unlikely to play a significant role in normal regulation of the elastic matrix. The demonstration by Murphy et al (1991) that human matrix metalloproteinases can degrade elastin may be more important in understanding the pathogenesis of diseases involving damage to the elastic fibres and increased elastin turnover. Gelatinase A and matrilysin were the most active enzymes with the gelatinase B being less efficient and stromelysin showing only a low elastinolytic activity (Murphy et al 1991).

Elastin is gradually lost with advancing age and little replacement occurs during which wound healing, which may explain the relative low elastinolytic activity.

1.5 Matrix Metalloproteinases

The degradation of connective tissue matrix is accelerated in many pathological process for example arthritides, periodontal diseases, tumour invasion and metastasis, but also occurs in the physiological remodelling associated with morphogenesis, growth, angiogenesis, cell migration, cervical softening, uterine involution and wound healing. The four major classes of proteinases are metallo-, serine, cysteine and aspartic defined by their active site residues. These are the most important enzymes in the remodelling process as they can degrade the protein components of most matrix macromolecules with the exopeptidases and glycosidases playing a secondary role.
The initial step in matrix degradation is an extracellular proteolytic process, which may range from fine modifications of glycoproteins associated with the cell to clipping of the cross-linked insoluble collagen and elastin fibres that are the basis of the matrix. The metalloproteinases are the most important enzymes in this extracellular step as they specifically degrade collagen (collagenase), are active at neutral pH and acting synergistically can digest the major macromolecules of connective tissue matrices. Serine proteinases that are active at neutral pH can also participate in this initial extracellular phase of the degradative process. Subsequent to this proteolytic phase the fragments generated may be phagocytosed by local cells for intracellular degradation or degraded further extracellularly.

1.5.1 The structure of matrix metalloproteinases

Matrix metalloproteinases are a family of enzymes derived from many types of mesenchymal cells, haemopoietic cells, including monocytes and macrophages and keratinocytes. These proteinases are capable of degrading at least one component of the extracellular matrix, are zinc and calcium ion dependent, secreted in a proenzyme form requiring extracellular activation, in vitro are activated by organomercurials or by treatment with trypsin or pepsin and their activity is inhibited by specific metalloproteinase inhibitors known as tissue inhibitors of metalloproteinases (TIMP). The primary amino acid sequences of both the latent and active forms of the enzymes have been determined and found to form 3 functional domains (fig 1.1). There is a high degree of sequence homology between different members of the matrix metalloproteinases family. The process of activation is accompanied by the cleavage of domain 1 ('pro domain') at a cysteine residue that is believed to play a role in maintaining the enzyme in the latent form (Docherty and Murphy 1990; Matrisian 1990).
Domain structure of the matrix metalloproteinases. The 'pre' domain is the signal peptide domain. The 'pro' domain is cleaved when the enzyme is converted to the active form. The 'catalytic' domain contains a conserved region that is believed to be the zinc-binding region. The 'hemopexin' domain is absent from matrilysin. The gelatinases contain a 'fibronectin' domain that has similarities to the collagen-binding domain of fibronectin. Gelatinase B also has a domain with sequence similarities to the α2 chain of type V collagen (Matrisian 1990).
By virtue of its content of histidine residues, which can co-ordinate the zinc ion on which metalloproteinase activity is dependent, domain 2 is thought to harbour the active site of the enzymes (Woessner, 1991). Both gelatinases contain a fibronectin domain in domain 2 that has similarities to the collagen binding domain of fibronectin (Matrisian 1990). The third, or carboxy-terminal domain, may be responsible for binding the enzymes to the connective tissue matrix; it is absent from matrilysin (Matrisian 1990; Allan et al 1991).

The matrix metalloproteinase family can be divided in three major subgroups; the interstitial collagenases, the gelatinases (type IV collagenases) and the stromelysins. The enzymes within a subgroup have similar primary structure with about 70% homology compared to 50% between the subgroups (Matrisian 1990, Docherty and Murphy 1990, Murphy and Reynolds 1993).

<table>
<thead>
<tr>
<th>Principle members of the Matrix Metalloproteinase family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
</tr>
<tr>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Interstitial collagenases</td>
</tr>
<tr>
<td>1 - $M_f 55,000$</td>
</tr>
<tr>
<td>2 - $M_f 75,000$</td>
</tr>
<tr>
<td>Gelatinases</td>
</tr>
<tr>
<td>1 - $M_f 72,000$</td>
</tr>
<tr>
<td>2 - $M_f 95,000$</td>
</tr>
<tr>
<td>Stromelysins</td>
</tr>
<tr>
<td>1 - $M_f 57,000$</td>
</tr>
<tr>
<td>(+ minor glyco-sylated forms of higher $M_f$)</td>
</tr>
<tr>
<td>2 - $M_f 57,000$</td>
</tr>
<tr>
<td>3 - $M_f$</td>
</tr>
<tr>
<td>Matrilysin</td>
</tr>
<tr>
<td>1 - $M_f 28,000$</td>
</tr>
</tbody>
</table>

(Matrisian 1990; Woessner 1991; Murphy and Reynolds 1993).
Two interstitial collagenases have been identified, a Mr 55,000 enzyme synthesised and secreted by connective cells and macrophages (Welgus et al 1985) and a Mr 75,000 enzyme stored in the specific granules of polymorphonuclear leukocytes. They have a specific action, cleaving the interstitial collagens types I, II and III at a single locus and have minimal activity on other matrix proteins. A Mr 72,000 gelatinase derived from mesenchymal cells (Murphy et al 1989a) and a Mr 95,000 gelatinase associated with macrophages (Hibbs et al 1987), polymorphonuclear leukocytes and stimulated connective tissue and tumour cells have been distinguished (Hibbs and Bainton 1989; Murphy et al 1989a; Murphy et al 1989b; Garbisa et al 1986). These are able to degrade denatured collagen (gelatin) (Murphy et al 1985b), as well as elastin and type IV, V, VII, XI collagens (Hibbs et al 1989). Two stromelysins have been identified and shown to be biochemically similar (Nicholson 1989). The identity of stromelysin-1 as the procollagenase activator and "matrix metalloproteinase 3" has been established (Murphy et al 1988). Matrilysin a lower Mr (28,000) metalloproteinase extracted from rat uterus (Woessner and Taplin 1988) is classified as the smallest member of the stromelysin group in which the third domain is missing (Matrisian 1990). The stromelysin sub-group has a broad substrate specificity, degrading ECM proteoglycans, laminin, fibronectin, gelatin, type IX collagen and the globular portion of basement membrane collagens (Matrisian 1990; Murphy and Reynolds 1993).

1.5.2 Synthesis and secretion of metalloproteinases

Matrix metalloproteinases are produced by many cell types including polymorphonuclear leukocytes (Murphy et al 1989), chondrocytes and endothelial cells (Murphy et al 1989), fibroblasts (Hipps et al 1991), macrophages (Hibbs et al 1987) and tumour cells (Gavrilovic et al 1985). Synthesis and secretion of metalloproteinases appears to be controlled by factors that alter gene expression and there is now evidence for an important role for cytokines, such as interleukin-1, and growth factors such as
basic fibroblast growth factor and platelet-derived growth factor (Murphy and Reynolds 1993) in this regulation. All these factors can induce collagenase or stromelysin gene expression in a variety of connective tissue cells (Frisch and Werb 1989; Overall et al 1989). Some of these stimulating factors are produced by the connective tissue cells themselves, others by macrophages and monocytes therefore autoregulation of connective tissue turnover can occur (Murphy et al 1990). Gelatinase production by human fibroblasts was found to be stimulated by transforming growth factor-β, as was TIMP, whereas collagenase synthesis was decreased (Edwards et al 1987, Overall et al 1989). Thus it is possible that gelatinase is controlled differently from the other metalloproteinases in some situations, which may be related to its different chromosomal localisation compared to collagenase and stromelysin (Spurr et al 1988, Huhtala et al 1990).

The synthesis of matrix metalloproteinases is almost always followed by their secretion. Studies of collagenase synthesis in human fibroblasts showed that intracellular enzyme appeared after 15 minutes and that after a further 15 minutes it was present extracellularly (Valle and Bauer 1979) which suggests there is no storage of intracellular enzyme. Hence the use of the ionophore monensin in cell and tissue cultures for immunofluorescent localisation studies to inhibit the secretion of the enzymes resulting in intracellular accumulation in the golgi apparatus and in secretory vesicles permitting identification of the cells actually synthesising the enzymes (Hembry et al 1985). Polymorphonuclear leukocytes, however, appear to store the enzymes in secretory granules. Secretion of metalloproteinases may be accompanied by the cleavage of a signal sequence of more than twenty amino acids from the N-terminal of the enzyme (Docherty and Murphy 1990).

Metalloproteinases derived from mesenchymal cells are secreted in a pro-form (Sellers et al 1978). The mechanism for their in vivo activation is still unclear, although there is evidence to suggest that plasmin, a proteinase, may be important. Plasminogen
activator specifically generates plasmin from its plasma zymogen, plasminogen, and the proteinase initiates the autoactivation of collagenase and stromelysins by a cleavage in the propeptide (Werb et al 1977, Gavrilovic and Murphy 1989). Additionally plasmin itself can degrade a number of connective tissue macromolecules, including fibronectin, proteoglycan core proteins, other glycoproteins, type IV collagen and fibrin (Moscatelli and Rifkin 1988, Gavrilovic and Murphy 1989). Other enzymes such as cathepsin B and L and trypsin have also been implicated in the activation of collagenase and stromelysin (Frisch and Werb 1989).

Once activated, stromelysin is required for complete activation of procollagenase which otherwise has a low activity. Stromelysin can elicit a 10-fold increase in collagenase activity at a molar ratio of 1:100 (Murphy et al 1987). Studies with cells that produce collagenase but not stromelysin in culture show that they are unable to degrade type I collagen films unless exogenous stromelysin is present (Murphy et al 1992). Neither plasmin nor stromelysin are efficient activators of gelatinases (Collier et al 1988) and the mechanism of activation of this group of enzymes in vivo remains unclear, with auto-activation being likely (Hipps et al 1991).

1.5.3 Inhibition of matrix metalloproteinase activity

The activity of matrix metalloproteinases may be controlled by non-specific and specific inhibitors. α₂-macroglobulin is the most effective non-specific inhibitor; occurring in human plasma it has a molecular weight of 780 kDa and consists of four identical polypeptide chains that can trap the enzymes within the molecule (Barret and Starkey 1973). Because of its large size, that precludes penetration within the matrix, its action is confined to the blood vessels (Cawston 1986). Therefore, to control metalloproteinase activity within the tissue stroma smaller molecules are required and this is accomplished by specific matrix metalloproteinase inhibitors, tissue inhibitors of metalloproteinases (TIMPs). Two members of the TIMP family have been described,
TIMP-1 and TIMP-2 (Ward et al 1991). The major form, TIMP-1 is a glycoprotein with a molecular weight of approximately 28 kDa which is synthesised and secreted by most connective tissue cells as well as by macrophages (Welgus et al 1985). Tight control of the active forms of all matrix metalloproteinase is given by TIMP specifically forming irreversible complexes with them (Cawston 1986) so that matrix degradation can only occur when the level of activated enzyme exceeds that of the inhibitor (Brown et al 1989). Recently endothelial cell stimulating angiogenic factor (ESAF) has been shown to be able to activate the collagenase-TIMP complex (Brown and Weiss 1988). TIMP-1 has also been shown to form a complex with the latent pro form of gelatinase B (Wilhelm et al 1989). This complex gives storage of TIMP-1 in the tissues and when required the inhibitor may dissociate from the weaker TIMP-1-progelatinase complex and combine with the higher affinity binding sites of the active enzymes (Ward et al 1991).

Human melanoma cells secrete TIMP-2 that has similar biochemical properties to TIMP-1 but is unglycosylated, has a different amino acid sequence and a lower molecular weight of 21kDa (Stetler-Stevenson et al 1989). TIMP-2 can bind the proform gelatinase A but not gelatinase B and it seems that much of the TIMP-2 and gelatinase produced by cells exists as a complex (Goldberg et al 1989, Murphy and Reynolds 1993).

1.5.4 Connective tissue degradation by matrix metalloproteinases

The extent to which metalloproteinases participate in the degradative action associated with morphogenesis, growth, remodelling and many pathological process is dependent on factors which modulate the expression of their proteolytic activity. In the normal and physiological turnover of connective tissue macromolecules the metalloproteinases are strictly controlled (Murphy and Reynolds 1985a) and no one proteinase prevails over the others in matrix turnover. A complex cascade of
proteolytic events is the best explanation of how matrix destruction takes place. Resorption is initiated by specific attack on matrix macromolecules by members of the family of metalloproteinases synthesised by stimulated connective tissue cells. However it seems that the accelerated breakdown of connective tissue occurring in pathological situations is mainly due to a failure of the normal regulation of the degradative process (Murphy and Reynolds 1993).

Although interstitial collagenase can cleave the resistant triple helix structure of the tropocollagen molecule, it is less able to do this in tightly cross-linked fibrils of mature collagen that are covalently bound with carbohydrate and have fibril interaction with glycoproteins in the extracellular matrix. It seems that for interstitial collagenase to act in vivo other non-specific proteases, such as cysteine and serine proteases, may be required (Harris and Krane 1974). These proteases could break the cross-links which exist between the non-helical regions of the tropocollagen molecules leaving the collagen molecules open to attack by interstitial collagenase (Gross 1981).

Interstitial and neutrophil collagenase cleaves the triple helix of the collagen molecule at a locus three quarters of the way between the amino and carboxy termini of the molecule. This occurs at a Gly-Ile bond in the $\alpha_1$(I) chains and at a Gly-Leu bond in the $\alpha_2$(I). The resultant degradation products are designated $\frac{3}{4}(TC_A)$ and $\frac{1}{4}(TC_B)$. The fragments quickly lose their triple helix conformation and the denatured products are able to be degraded further by gelatinase and other proteases (Harris and Krane 1974).

Matrix metalloproteinases are involved in the degradation of other collagen molecules. Notably in the wound healing process is the degradation of types IV and V collagen, which are present in basement membrane. Breakdown of basement membrane is necessary for capillary remodelling and growth during angiogenesis in the third phase of wound healing. Type IV collagen can be degraded by the action of many
proteinases in vitro, including pepsin and plasmin (Crouch et al 1980), and in vivo both enzymes are required for degradation of the insoluble basement membrane. Both gelatinases specifically cleave type IV collagen at a single site within the helical portion of the molecule to produce fragments resembling the 3/4 - 1/4 products produced by interstitial collagenases in the degradation of type I collagen (Murphy et al 1989b). The serine proteinases synthesised by polymorphonuclear leukocytes, elastase and cathepsin G can also degrade type IV collagen by attack on the non-helical regions (Davies et al 1978, Pipoly and Crouch 1987). Stromelysin too can degrade type IV collagen attacking the non-helical regions, in the cross-linked regions of the molecule although its activity is much less potent than that of gelatinase (Murphy et al 1991).

Stromelysin's major action is the degradation of proteoglycans efficiently cleaving the protein core at multiple sites, acting over a broad pH range. In comparison collagenase and gelatinase have a limited action against the protein core whereas in vitro the serine proteinases, elastase and cathepsin G have been shown to cleave the core protein at multiple cleavage sites (Galloway et al 1983, Nguyen et al 1989). The glycosaminoglycan side chains are degraded intracellularly by the action of lysosomal endoglycosidases, exoglycosidases and sulphatases. There is no evidence for cleavage of glycosaminoglycans at extracellular sites except in the case of heparan sulphate, degraded by heparatinases produced by platelets (Oosta et al 1982, Poole 1986).

The normal turnover of elastin in tissues is very slow and may be mediated by the metalloproteinases produced by local cells. Gelatinase A and matrilysin are the most active enzymes for breakdown of insoluble elastin, gelatinase B is less efficient and stromelysin has the lowest elastinolytic activity (Murphy et al 1991). In situations of more rapid breakdown, such as emphysema, more potent proteinases may be involved, including cathepsin L from macrophages and leukocyte elastase from polymorphonuclear leukocytes (Senior et al 1989).
Extracellular glycoproteins, especially the extensively distributed fibronectin and laminin, are now well characterised and their roles defined (Yamada 1989, Martin and Timpl 1987), but the process of degradation of these molecules is still unknown. The only metalloproteinase participating in the degradation of the glycoproteins is stromelysin (Galloway et al 1983). Glycoproteins are more susceptible to the action of many serine proteinases, such as plasminogen activator (Quigley et al 1987), elastase and cathepsin G (Heck et al 1990). Plasmin is thought to play the most significant role in the removal of the glycoprotein components of the matrix (Murphy and Reynolds 1993).

The metalloproteinases through their ability to degrade most components of the ECM both extracellularly and at physiological pH are likely to have a fundamental role in any situation in which connective tissue turnover is of importance, as in colonic anastomosis where there is evidence of a higher collagenolytic activity than in other segments of the gastrointestinal tract. However, there are no clinical or experimental documentation of the role of gelatinase, stromelysin and their inhibitor TIMP and many aspects of collagenase activity need to be clarified.

1.6 Animal models of colonic anastomosis

To study the healing of colonic anastomosis an animal model is necessary because it is crucial to investigate early events after surgery, when neither samples of tissues nor total anastomoses can be obtained from humans.

Most frequently colonic anastomotic healing has been investigated in rats because of the easy housing and low costs, which makes it possible to study a larger number of animals. Many groups have worked with rat models for example Cronin et al (1968), Irvin and Hunt (1974a), Irvin (1976, 1978), Jiborn et al (1978, 1980a), Stromberg and Klein (1982), Jonsson et al (1983, 1985, 1986, 1987), Blomquist et al
1984, 1985), Hogstrom (1986) and more recently Mastboom, Hendricks and de Boer (1989) and Marstens and Hendricks (1991). Rats also have the advantage of a colonic anatomy similar to man with a small caecum and a short left colon passing down to the rectum but their physiology and pharmacology are different from human. However the small size of the rat makes studies of surgical techniques difficult and therefore researchers have used larger animals such as pigs, cats and dogs.

1.6.1 The rabbit model

Rabbits have the advantage of easier housing and lower costs than cats and dogs but are large enough to allow the execution of different anastomotic techniques which are not possible in the rat. There are some anatomical differences as rabbits are herbivores, with a large caecum and right colon, but its left colon, similarly to man, has a complete coat of longitudinal muscle that merges into the rectum which has an extraperitoneal segment before terminating in the anus. This anatomical configuration permits the creation, with safety, of an intraperitoneal colorectal anastomosis in the most distal colonic segment allowing the characteristics of the healing process in the distal colon to be examined. Physiologically and pharmacologically the rabbit colon has been shown to resemble the human more closely than the rat (Percy et al, 1989). As the principal aim of this project was to study metalloproteinases it was essential to have a rabbit model as previous investigations have shown that collagenolytic activity can be measured in rabbits but not rats (Gavrilovic et al 1987). Indeed much of the previous work on MMP has measured collagenolytic activity in the rabbit colon, for example Hawley (1970 a. b), Jayaraj et al (1983), Lunstedt et al (1984), Hesp et al (1984, 1985), Hendricks et al (1985) and Lewin et al (1986). Immunocytochemistry was chosen as the method of investigation as the MMPs are only expressed on demand in inadequate quantities for direct biochemical measurements. Immunocytochemistry also allows to localise the tissue distribution of MMPs. Antisera are at present only
available to rabbit and do not give sufficient cross reactivity with MMPs of other species, that can be of use.

1.6.2 Suturing material and technique

Since the beginning of this century animal models have been used to search for the best suture material for surgery. Halsted (1913), Whipple (1933), Shambaugh and Dunphy (1937) and Elkins (1940) in clinical and experimental studies compared suturing with catgut to silk and showed that silk produced greater tensile strength and lower wound infection rates because it produced less inflammation than catgut. Madsen (1953a, 1953b, 1958) compared 12 different suture materials in rabbit abdominal wounds and concluded that absorbable suture materials cause a marked exudative tissue reaction and a delayed formation of collagen with a decreased tensile strength.

The choice of suture material in colonic anastomosis is based on very few experimental studies. Comparative studies in colonic anastomosis between cotton and chromic catgut (Fellows et al 1951), chromic catgut and reconstituted collagen sutures (Adler et al 1967), polyglycolic acid and chromic catgut (Munday and McGinn 1976), catgut, braided silk, polyglycolic acid, proline and dacron (Lord et al 1978), indicated that any type of suture used for intestinal anastomosis produces inflammation during the lag period of wound healing and no one suture material has been shown to be consistently superior.

A number of experimental studies have compared colonic anastomotic healing with inverted and everted suturing. A few reports indicate an advantage in eversion of the anastomosis (Getzen et al 1966 and Ravitch et al 1967), and most showed preference for the inverted technique (Hamilton 1967, Loeb 1967, Hargreaves and Keddie 1968, Trueblood et al 1969, Gill et al 1969 and Irvin and Edwards 1973). These studies have shown that inversion produces narrowing but promotes earlier
healing by restoration of mucosal continuity and that the anastomoses are more resistant to disruption. Eversion anastomosis results in more local complications, namely adhesions and dehiscence and is now rarely used clinically. A two layer inverting anastomosis reduces mucosal blood flow (Chung 1987) which increases the degree of stenosis (Sacko and Wangensteen 1951, McAdams et al 1969, Irvin and Edwards 1973). Instead a single layer closure in colonic anastomosis should allow better healing provided that the amount of tissue inverted by the suture line is minimised to avert compromising mucosal blood flow and luminal diameter (Koruda and Rolandelli, 1990).

In a pilot study Hawley (1969) observed a high incidence of intestinal obstruction using continuous sutures for left colonic anastomosis in rabbits and therefore used interrupted sutures for further work. Jiborn et al (1980b) compared a single layer of continuous inverting suture with a single layer of interrupted inverting suture in the rat colon. They observed a decreased collagen concentration in the anastomotic region extending 2 to 3 cm proximal to the anastomosis but only 0.5 cm distal to it. In animals with continuous suture the return to a normal collagen concentration was slower than in those with interrupted sutures. Colonic dilatation and faecal stagnation proximal to the anastomosis were observed in some animals with continuous suture. This was associated with an enhanced rate of collagen turnover and a greater breakdown of collagen in the colonic wall than in animals with uncomplicated healing. These findings are consistent with the clinical observations reported by Goligher et al (1970) and Irvin and Goligher (1973) that faecal loading carries a high incidence of anastomotic dehiscence in colonic surgery.
1.6.3 Intestinal obstruction and colonic anastomosis

There are few experimental studies in which colonic obstruction has been examined. Atik et al (1960) were one of the first to study primary anastomosis of the obstructed colon. They created a complete obstruction of the large bowel dividing the mid-colon of dogs between clamps and closing the ends with Parker-Kerr sutures. After one week of obstruction they compared primary resection and anastomosis with and without antibiotics. Antibiotics were given intraluminally, intraperitoneally and intravenously. They concluded that the local instillation of antibiotics in the presence of obstruction did not sterilise the colon or significantly reduce the high mortality of primary resection and anastomosis in an obstructed left colon.

The effects of bowel distension on intestinal blood flow in the small and in the large bowel were studied by Boley et al (1969), Ruf et al (1980) and Shikata et al (1983) which produced acute and complete obstruction by tying umbilical tapes around the bowel. They found a fall in intestinal blood flow with intra-luminal pressures above 30 mm/Hg. Coxon et al (1984) induced progressive fibrosis with a sheet of silastic rubber placed around the rectum in mini-pigs. Papanicolaou et al (1985, 1989) produced in dogs an incomplete obstruction by tying a tape around the bowel until reduced to half its diameter. In these models of chronic large bowel obstruction unlike in the acute obstruction model, the blood flow in the bowel proximal to the site of obstruction was increased. An acute model may not be representative of the obstruction produced by malignancy and it is likely that a period of chronic large bowel obstruction precedes the acute episode.
Leaper (1983) studied the healing of colonic anastomosis in rats by angiography. After 72 hours of colonic obstruction he found an increased risk of anastomotic failure because of delay in the formation of new vessels and in their growth across the anastomosis. He concluded that primary anastomosis in acutely obstructed colon is contra-indicated because of the risk of delayed healing.

Stromberg and Klein (1982) studied the effect of intestinal obstruction on collagen dynamics after producing a partial obstruction in the small bowel by placing a loose circumferential band around the terminal ileum. They demonstrated that obstruction produced a fivefold increase in total collagen. Throughout the study pre-existing pre-obstruction collagen was metabolically stable with no breakdown or demonstrable remodelling. New collagen formation was rapid and mostly occurred close to the site of obstruction and therefore little collagen degradation took place and only of new collagen.

Jiborn et al (1978) studying colonic anastomosis in rats after left colonic resection observed that continuous sutures produced partial obstruction at the anastomosis in some animals. The resultant faecal loading caused a high incidence of dehiscence and a marked decrease of collagen concentration in the anastomosis. Tornqvist et al (1988) studied the effect of stenosis on collagen metabolism in the colonic wall. They created a model of colonic obstruction by placing a silicon ring with an inner diameter of 6.5mm around the colon between two marginal blood vessels approximately 2.5cm above the peritoneal reflection. With this technique they caused marked faecal loading and some stagnation in the stenosed part. After four days of obstruction the stenosis was resected and an anastomosis formed. They observed that in the obstruction only group at the day of operation the collagen synthesis in the proximal anastomotic segment was significantly higher than that of the distal one. As collagen content was equal in both segments they concluded that in the proximal segment there was also an increased collagen lysis. Collagen metabolism following an anastomosis either alone or
preceded by obstructing the colon and resection of the obstructed segment was also studied. No difference in collagen content was measured between the groups, however in the obstructed group collagen synthesis proximal to the anastomosis, but not at or distal to it, was twice as high implying that collagen lysis must also be occurring at a much greater rate in this region. The major difference between the groups was in the complication rate which was 2 per cent in the anastomosis only group compared to 27 per cent in the obstruction and anastomosis animals. This study shows that an obstruction does have a profound effect on collagen dynamics with both synthesis and degradation being affected.

1.6.4 Intestinal ischaemia

Early knowledge on ischaemic bowel disease emanates from descriptions of mesenteric vascular occlusion, first described in 1843 by Tiedemann (Laufman et al 1964) and as early as 1904 Jackson et al published a study of 214 clinical cases. In a review by Laufman et al (1964) they cite other experimental studies from the last century, mainly by Germans, studying occlusions of the mesenteric vessels (von Beckman 1858, Cohn B 1860, Conheim J F 1872, von Heller A 1870, Howse H G 1878, Karcher J 1897, Tangl F and Harley V 1895, Welch W H 1887).

Very little was known at the end of the century about ischaemia of the colon, most reports referring to superior mesenteric artery occlusion and small bowel ischaemia. In the 1950s there were numerous reports of iatrogenic ischaemic injuries following aortography and after ligation of the inferior mesenteric artery during resection of aortic aneurysms (Moore 1954, Movius 1955, McKain and Shumacker 1958, Smith et al 1958). The study of colonic ischaemia developed faster in the 1960s with experimental and clinical investigations by groups in the U.S.A. and in England. Boley et al (1963) and Schwartz et al (1963) from New York reported five clinical
cases of reversible vascular occlusion of the colon and established the basis for clinical diagnosis, with emphasis on the radiological appearance of pseudotumours in the colon. In an experimental study in dogs, by ligating either the major arcade to the bowel segment or all the vessels running between the arcade and the colon, they reproduced the radiological changes seen in man and demonstrated submucosal haemorrhage with associated intraluminal bleeding and pericolic inflammation, which they believed caused the pseudotumours. Marston (1964) in London demonstrated that when the superior mesenteric artery is suddenly occluded instant necrosis of the small bowel occurs, starting at the tips of the villi and extending outwards towards the serosa. The minute vessels of the intestinal mucosa rapidly suffer anoxic damage and lose their power to retain plasma. He demonstrated that although there is no arterial flow the portal pressure is maintained by inflow from the coeliac and the mesenteric routes which is the cause of plasma exudation into the wall and lumen of the bowel. He attempted to reproduce ischaemic lesions in the dog colon by occluding small intestinal vessels for varying periods of time but without success, probably because the vasa recta of the dog communicate freely. Boley et al (1965) utilised another model of colonic ischaemia injecting microspheres into the caudal mesenteric artery. They obtained pathological changes ranging from superficial mucosal ulceration to total necrosis which were identical to those seen in the human intestine.

Clinical and experimental investigation of ischaemic bowel by Boley and Marston established the pathophysiology and the clinical course of the disease, however, they made no reference to the risk of ischaemia on colonic anastomosis although its implication was clear.

Attention next focused on methods of determining bowel perfusion and viability. Clinical criteria such as arterial pulsation, colour of the bowel or peristalsis are poor guides and have a low sensitivity (Gorey 1980) hence the need for alternative measurements. Bussemaker and Lindeman (1972) compared the surface temperature of
the bowel and electromyography to clinical criteria in dogs and Katz et al (1974) compared electromyography, pH determination and tetrazolium analysis of the mucosa. These measurements were time consuming, had low specificity and needed to be used collectively to be of value. Other methods tested were radioactive microspheres (Zarins et al 1974), polarographic oxygen electrode (Piasecki 1981), mucosal albumin clearance (Parks et al 1982b), surface oximetry (Locke et al 1984), infrared photoplethysmography (Pearce et al 1987), intramural pH (Senagore et al 1990), hydrogen gas clearance (Oohata et al 1990) and tissue oxygen tension (Sheridan et al 1992) but none has been of general acceptance either because of poor sensitivity or complexity for routine use.


Despite much work on blood flow measurement its role in the healing process has not been adequately explored. Some indirect evidence of the importance of a normal blood flow in wound healing was obtained by the measurements of oxygen and carbon dioxide tensions in healing of skin wounds in rats. These studies have shown that an increase in pO$_2$ in inspired air results in accelerated collagen synthesis and increased wound tensile strength while addition of excess CO$_2$ to the inspired air was associated with a reduced wound tensile strength (Stephens and Hunt 1971, Hunt and Pai 1972).
Kirk and Irvin (1977) studied the role of oxygen therapy on the healing of skin wounds and colonic anastomosis in rats breathing air compared to rats breathing 50% oxygen. Wound healing was assessed by measurements of breaking strength, colonic bursting pressure and wound collagen levels after seven days of treatment. They found no difference between the groups of rats for either skin or colonic wounds and concluded that oxygen therapy had no effect on wound healing. Cohen et al studied the effect of ischaemia on colonic wound healing with and without antibiotic preparation of the bowel. Anastomotic healing in unprepared ischaemic rat colon was severely impaired with an 83% dehiscence rate while no anastomotic dehiscence occurred in those rats receiving antibiotics. Cohen et al (1985) suggested that the bacterial flora were crucial for the progression of intestinal ischaemia to gangrene and perforation.

In one of the few investigations of rabbit colonic healing Shandall et al (1985) studied the effect of hypoxia. They created a model of ischaemia in the sigmoid colon by tying all the small vessels arising from the posterior mesenteric artery for approximately 7 cm. A specially constructed Clark oxygen electrode was used to measure oxygen tension in the ischaemic segment, which ranged from 0 mmHg at the centre of the segment to 60 mmHg at its border. Guided by the Clark electrode they performed anastomoses at varying oxygen tensions and observed the occurrence of either "major leaks" (death due to anastomotic failure) or "minor leaks" defined as a localised collection at ten days post-operatively. All anastomoses formed at a perianastomotic oxygen tension less than 20 mmHg developed major leaks; above 30 mmHg they observed 10% major leaks and 30% minor leaks; above 40 mmHg only 5% minor leaks were seen. These results may be explained by the fact that below 20 mmHg energy metabolism in wounds is impaired and proliferation of fibroblast is arrested below 25 mmHg (Kivisaari et al 1975, Silver 1973).
The same group (Sheridan et al 1987) used the Clark electrode to measure tissue oxygen tension in the human colon and correlated the results with anastomotic healing. Measuring oxygen tension in various segments of the human colon they found the highest levels in the sigmoid colon, transverse colon and rectum and the lowest in the descending colon and caecum. Studying a group of fifty patients they observed five anastomotic leaks following anterior resection. In four of them the oxygen tension in the colon which had been mobilised for anastomosis was relatively low. In the fifth, although the oxygen tension was normal, the bowel had been damaged by previous irradiation. These findings are in support of the role of relative tissue hypoxia in colonic anastomotic failure. A modified Clark electrode was also used by Foster et al (1985a) to measure tissue oxygen tension in the rat colon and small bowel. The oxygen tension was lower in the colon than in the small bowel and fell significantly following 10 per cent blood loss. Assuming that hypovolemia leads to tissue hypoxia and this in turn produces impaired anastomotic healing they suggested that adequate intravenous fluid replacement during colonic resection and anastomosis is a pre-requisite for successful healing.

Forrester et al (1981) studied colonic blood flow in patients undergoing colostomy closure and found evidence of an abnormal blood flow associated with fistula formation. Billings and Leaper (1987) using laser Doppler velocimetry measured blood flow in colostomies and demonstrated a median level of 19 units at one week increasing to a median of 44 units at eight weeks. They concluded that laser Doppler flowmetry is a simple, non-invasive method of measuring colostomy blood flow and from their results advised that colostomy closure should be delayed to avoid fistulation. Their findings are supported by the clinical observations of Thomson and Hawley (1972). They reviewed 139 patients who underwent closure of a loop transverse colostomy and found that four of 38 patients whose colostomies were closed at less than one month developed fistulas. No fistulas were observed in 101 patients...
that had their colostomies closed after one month. Similar results were reported by Garnjobst et al (1978) and Foster et al (1985b).

In the last ten years many investigators have examined the role of oxygen free radicals in ischaemic bowel. Using models of ischaemia followed by reperfusion or low flow states during arterial hypotension in the small bowel, early mucosal damage (Parks et al 1982a, Parks and Granger 1986), the pathophysiology of the damage (Granger et al 1981, Parks et al 1982b, Parks and Granger 1983, Parks et al 1984, Bulkley et al 1985, Grisham et al 1986, Cassuto et al 1979, Hogstrom and Haglund 1986, Haglund et al 1987, Leung et al 1992) and the action of trypsin inhibitors (Parks et al 1985), have been studied extensively and is worth quoting but its detail is as yet not relevant to the subject under investigation.
CHAPTER 2

PURPOSE AND PLAN OF THE STUDY

The most feared complication of a colonic anastomosis is dehiscence. In a recent and comprehensive multicentre trial using strictly defined clinical criteria the anastomotic dehiscence rate ranged from 0.5% to more than 30%, with an average of 13%, and was higher in anterior resection of the rectum. This was associated with an increased mortality from 7.1% to 22% and prolonged hospital stay from 25.4 to 45.7 days (Fielding et al 1980). The overall experience reported by others have conformed with these observations (see 1.3.1). Allowing for the surgeon's technical skill and his perfection of the technique in determining the outcome of an anastomosis (Fielding et al 1980) other factors play a part. These have been identified by Morgenstern et al (1972) as due to systemic conditions and included advanced age, malnutrition, coagulopathy, steroid therapy, diabetes, uraemia and they considered performing an anastomosis in an inflamed colon and in a contaminated peritoneal cavity as an important local risk factor. Schrock (1973) recognised anaemia, irradiated bowel, residual carcinoma at the resection margins, sepsis, intraoperative hypotension and massive haemorrhage as other unfavourable factors.

However, even with careful selection and preparation of the high risk patient and despite developments in anaesthesia, bowel cleansing, antimicrobial prophylaxis and meticulous operative technique by avoiding tension and devascularisation of the bowel and securing haemostasis, dehiscence remains an alarming complication in colorectal surgery. Fielding et al (1980) concluded that with a standard surgical technique and in the absence of any of the recognised predisposing factors an anastomotic failure is still likely to occur in 5% of patients. Therefore anastomosis performed under "ideal" conditions may still dehisce for no apparent reason.
The submucosa with its large collagen component has been recognised as the most important layer in maintaining the strength of the bowel wall during healing (Halsted 1887). With this knowledge early studies on intestinal anastomosis attributed anastomotic dehiscence to excessive activity of collagenase since it is the enzyme that specifically degrades collagen. Cronin (1968) showed that collagen levels are significantly decreased in the initial period of healing and Hawley (1970b) demonstrated a high collagenolytic activity in the distal colon compared to the rest of the gastrointestinal tract. Dunphy (1970) in a series of experiments found that up to 40% of normal collagen is lost in the first four to six days after anastomosis and was associated with diminished mechanical strength. These findings were later confirmed by Jiborn et al (1980a) and Jonsson et al (1985) who also reported variation in collagen concentration in the vicinity of an anastomosis. This loss of collagen was attributed without convincing evidence to collagenase activity.

Measurement of collagenase within tissues is difficult because it is secreted only on demand and active enzyme is strictly regulated by its inhibitor so that inadequate quantities are available for its measurement by direct biochemical methods. However by in vivo studies its distribution and that of other matrix metalloproteinases (MMPs) in the colon during healing can be determined by immunolocalisation using specific antisera. Chowcat et al (1988) in a rabbit model demonstrated for the first time, by immunohistochemistry, the presence of collagenase and TIMP-1 in colonic anastomosis, but the role of other MMPs was not examined.

MMPs are a family of enzymes secreted by mesenchymal, endothelial and haemopoietic cells that contain Zn\(^{2+}\) at the active site and are Ca\(^{2+}\) dependent. They are active at neutral pH and are all inhibited by specifically secreted inhibitors, tissue inhibitors of metalloproteinases (TIMPs). The collagenases cleave collagens types I, II, and III at a single locus; gelatinases cleave denatured collagens (gelatins) and type IV collagen; stromelysins are more general proteinases, cleaving proteoglycan core protein,
fibronectin and type IV collagen. Thus, combined these enzymes can degrade all the macromolecular components of connective tissue.

The purpose of this investigation was to study the possible role of MMPs and TIMP-1, in colonic healing, using immunohistochemistry to localise these enzymes in colonic anastomosis. Chowcat et al (1988) restricted their studies to the suture line after a standard resection and anastomosis. They found that collagenase and TIMP were confined within this area and were involved in the breakdown of the everted tissue in a synchronously controlled remodelling process, but there was no evidence of excessive collagenase activity. Hence this study was designed to detect all MMPs and TIMP in tissue samples around and away from the anastomotic region in a normal colon and in a colon that had been subjected to obstruction or ischaemia. Intestinal obstruction interferes with blood flow to the bowel and changes in collagen content have been reported with colonic dilatation (Jiborn et al 1980b) and obstruction per se also interferes with collagen dynamics. Ischaemia would allow examination of MMPs and TIMP in a) unhealthy necrosed tissue and b) the residual colon after the injury. Irvin and Hunt (1974b) had shown that intra-abdominal trauma in the vicinity of a colonic anastomosis had a significant effect on colonic healing. Although it was proposed to examine colonic anastomosis in the presence of peritonitis, this study was not approved by the licensing authority in the Home Office.

A rabbit model was chosen because specific antisera to metalloproteinases are only available for this animal. Due to the narrow diameter of the rabbit colon an inverted or a continuous sutured anastomosis can cause obstruction which would influence collagen dynamics (Jiborn et al 1980b, Stromberg and Klein 1982, Tornqvist et al 1988). Chowcat et al (1988) used an everted anastomosis to avoid luminal obstruction. In this study a single layer of extramucosal interrupted silk sutures was used instead in order to construct an anastomosis nearest to the conventional anastomosis used in surgical practice.
The distribution of the metalloproteinases and TIMP-1 was examined both in the anastomotic region and in tissue from around the colon after (i) transection of normal colon and resection following (ii) colonic obstruction and (iii) colonic ischaemia.

In the first group the distal colon was transected at a determined site (Chowcat et al 1988) and an anastomosis carried out. Because of reported collagenase activity after laparotomy (Hawley 1969), also included were animals killed 24h and 3 days after a sham operation; where after laparotomy and manipulation of the distal bowel, but without transection or anastomosis, a colonic segment was resected from the region of the standard site for examination.

In the second group intestinal obstruction was modified from the method described by Tornqvist et al (1990a), placing a silastic ring around the colon at a standard site as defined by Chowcat et al (1988). The model of Tornqvist was created in rats and to adapt the model to the rabbit, a series of experiments was carried out to determine the ideal diameter of the silastic ring and the time required to cause obstruction with proximal dilatation without causing bowel perforation. Having established the model the animals were subgrouped to examine changes after the release of the silastic ring and after resection of the obstructing segment and anastomosis as well as the effect of the silastic material on the colon.

In the third group ischaemia was induced by ligating and dividing the small vessels arising from the posterior mesenteric artery as described by Shandall et al (1985). A series of experiments was carried out to determine the extent and the duration of devascularisation required to develop a model of ischaemia that satisfied clinical and histological criteria. At different periods, the ischaemic bowel was resected and an anastomosis performed.
The animals were sacrificed at different times after their definitive operation for tissue sampling. An indirect immunofluorescent technique was applied to tissue from the anastomotic area and from segments distal and proximal to the anastomosis using antisera to collagenase, stromelysin and gelatinase and to their inhibitor, tissue inhibitor of metalloproteinases (TIMP-1). Sections were also stained for haematoxylin and eosin (H&E) to allow correlation with the histological changes.
CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Female New Zealand White Rabbits (Froxfield Farm, Hampshire, U.K.) from 1.5 to 2.5 kg were used for all experiments. Animals were housed in individual cages in an animal house with free access to water and a standard rabbit pellet diet (Special Diet Service - SDS, Cheshire, U.K.). The rabbits were anaesthetised by intramuscular hypnorm (Janssen Pharmaceutical Ltd., Grove, Oxford, U.K.) 0.3 mg/kg body weight and intraperitoneal diazepam (Pharma Hamelin G.m.b.H. W. Germany) 2 mg/kg body weight. Anaesthesia was maintained with hypnorm (0.2 mg/kg) every 45 minutes where necessary. The animals were sacrificed by an overdose of pentobarbitone sodium (Sanofi Animal Health Ltd. Watford, Herts, U.K.) 200 mg/ml, 0.75 mg/kg given via the marginal ear vein.

All chemicals were supplied by Merck Limited, Poole, Dorset, U.K. and of at least AnalaR grade unless otherwise stated. All antisera to metalloproteinases and TIMP-1 were developed and supplied by Strangeways Research Laboratory, Cambridge, U.K.

3.2 Methods

The models were developed for the purpose of studying matrix metalloproteinases and TIMP-1 distribution following resection and anastomosis of (1) a normal colon, (2) an obstructed and (3) an ischaemic colon. The methods employed to develop these models are described separately in detail in the following chapter.
3.2.1 Operative technique for colonic anastomosis

No bowel preparation was carried out. The abdominal wall was cleaned with liquid detergent and the operation was carried out with clean but not sterile instruments. A lower midline incision, 5cm long allowed access to the distal colon. The anastomosis was located in a region previously established by Chowcat (1988), five to eight centimetres above the peritoneal reflection. Above 8cm the rabbit posterior mesenteric artery runs in the mesentery at a distance of approximately one cm from the bowel wall, with infrequent branches to the bowel. In the distal three or four centimetres, the vessel approaches the colon wall closer and then quite abruptly begins to give more branches, only two or three millimetres apart, so that the colon below this point has an abundant blood supply (fig 3.1). This particular anatomy makes it possible to transect or resect the bowel in an avascular segment of the mesentery proximal to the point of vascular branching without the need to ligate the vessels and to form an anastomosis in a segment which is rich in blood supply. This has been defined as the "standard site" for the different studies in this work. However in the obstruction and the ischaemia models it was necessary to resect a longer segment of colon to ensure excision of all the unhealthy tissue and this required ligating a few blood vessels (see 4.2 and 3). A single layer extra-mucosal interrupted 6/0 silk sutures (Ethicon, Edinburgh, Scotland) were placed near the border of the cut edge of the bowel, on average 12 sutures were inserted. The peritoneal cavity was irrigated by the instillation of 20ml of saline before the abdomen was closed in two layers with continuous 2/0 dexon sutures (Cyanamid, Hampshire). The rabbits were allowed free access to food and water after the operation.

3.2.2 Sampling and preparation of tissue

At different scheduled times ranging from zero hours to seven days after anastomosis, the animals were sacrificed. The abdomen was opened and the site of operation inspected. After freeing any adhesions the distal colon was examined for signs
of leakage. Segments were excised from the anastomotic region and other sites throughout the colon as appropriate for the individual model and study (see chapter 4).

The segments of colon were opened at the mesenteric border, cleared of any faeces and then immersed in Hank's balanced salts solution (Appendix). Each piece of colon was washed by soaking and intermittent agitation in five changes of Hank's solution, the first three containing 10 times the usual concentration of antibiotics. The segments were transferred to a tissue culture hood whilst in the fifth wash and dissected with sterile instruments. Each segment of colon was cut longitudinally into strips, 3mm wide, and adjacent strips were either processed immediately or placed into short term tissue culture. One strip from each segment of colon was fixed in formol saline for processing for routine histology.

The strips of tissue for short term culture were placed in separate 3.5cm diameter wells of a six well tissue culture plate (Nunc, Denmark), each containing 4ml of Dulbecco's Modification of Eagle's Medium (ICN FLOW, High Wycombe, Berks., U.K.). Culture for three hours was chosen as previously it had been shown that there was no difference in the distribution of collagenase or TIMP secreting cells after three or six hours culture, indicating that the penetration of monensin into the tissue was complete within three hours and that the amount of enzyme or TIMP synthesised within this period resulted in sufficient accumulation in the golgi apparatus to improve intracellular visualisation (Chowcat, 1988). Also, restaining of some sections with haematoxylin and eosin, showed marked deterioration of the tissue after six hours compared to three hours culture and tissue cultured for 24 hours underwent so much disintegration that sections were difficult to cut and orientation of the tissue was sometimes impossible. In this study, therefore, culture with monensin was for three hours to restrict tissue damage and maintain sufficient structure to be able to locate the enzymes and TIMP-1.
Each 100ml of media contained supplements of 20mM HEPES, 1ml foetal calf serum (ICN FLOW, High Wycombe, Berks., U.K.), 1ml of 200mM glutamine (ICN FLOW) NaHCO₃, antibiotics (2ml of 100 units/ml penicillin, 2ml of 100ug/ml streptomycin, Sigma, St. Louis, Missouri, U.S.A), an anti-fungal (0.1ml of 2.5ug/ml amphotericin-B, Sigma, St Louis, Missouri, U.S.A) and monensin (5µM) (Sigma, Poole, Dorset, U.K.). Monensin is a monovalent ionophore which prevents the translocation of secretory proteins (Ledger et al 1980, Nagase et al 1983) allowing their accumulation in the Golgi apparatus. The cells actively synthesising enzymes and inhibitor can be readily identified microscopically by the secretory granules (Hembry et al, 1985, 1986). Culture plates were incubated in a humidified atmosphere at 37°C for three hours. The tissue was then processed in the same way as uncultured tissue.

Each strip of colon, cultured or not, was orientated on a nitrocellulose filter (Whatman Ltd, Maidstone, England) and embedded in 7 per cent gelatin in 0.9 per cent saline containing 0.2 per cent sodium azide as an antibacterial agent. The strip was orientated so that sections were cut at 90° to the plane of the bowel wall to show a longitudinal section of tissue. Tubes containing tissue and gelatin were frozen for 90 seconds in liquid nitrogen and stored at -20°C until required. Whenever a block was cut all the primary antibodies were applied and the blocks were cut two or more times to determine the distribution pattern of MMPs and TIMP-1 throughout the circumference of the bowel.

3.2.3 Histological examination

Tissue was prepared for haematoxylin and eosin staining (H&E) to allow matching MMPs and TIMP-1 staining with the histological appearance. Fresh tissue was placed immediately in formol saline for 24h each tissue was cut into 2cm thick piece, put into cassettes and processed by dehydration through 70% alcohol overnight, 90% alcohol for 30 min, four changes of 100% alcohol each for 1h, CNP30 for 1h, CNP30
overnight, wax for 1h and wax overnight. Tissue pieces were placed in molten wax and allowed to cool to form tissue embedded in wax and then sections cut. Dried sections were rehydrated through CNP-30, 100%-alcohol and 70% alcohol and washed in tap water. Stained with Harris's haematoxylin for 7 minutes, washed in tap water for 5 minutes, then differentiated with 1% acid alcohol for 5 to 10 seconds until the nuclei were blue and the stroma light blue. After washing in tap water until sections were blue, they were stained with 1% eosin for 5 minutes then washed in tap water for few seconds and dehydrated through alcohols, each for a few seconds 70%, alcohol two changes each of 100% alcohol and CNP30, then mounted with DPX and visualised by standard light microscopy (Olympus, Japan).

3.2.4 Immunolocalisation

To investigate the localisation of the matrix metalloproteinases and their natural inhibitor, tissue inhibitor of metalloproteinases (TIMP), specific antibodies to rabbit collagenase, gelatinase, stromelysin and TIMP were used. Their distribution was visualised by the application of a second antibody labelled with fluorescein isothiocyanate (FITC) (Hembry et al 1986). This technique gives not only the distribution of the matrix metalloproteinases and TIMP-1 but is sufficiently sensitive to locate the granules of enzyme and inhibitor in the cells as they are synthesised. The accuracy of information obtained from such a technique depends on the specificity of the antibodies employed (Woolley et al 1980).

Anti-collagenase IgG was produced by the immunisation of sheep with a purified preparation of rabbit collagenase and the specificity of the antibody was determined by double immunodiffusion, electrophoretic immunoblotting and immunoprecipitation (Hembry et al 1986). This antibody was characterised in experiments with collagen fibrils, cultured rabbit chondrocytes and post-partum rabbit uterus. It was shown to react
with both active and latent collagenase and to identify extracellular enzyme, collagenase bound to collagen and secretory granules within cells.

Antiserum to rabbit bone gelatinase was raised by the immunisation of sheep with a purified rabbit gelatinase from bone culture medium (Murphy et al 1989b). It was shown by immunoblotting to detect both gelatinase A, produced by connective tissue cells from rabbit, pig, human and mouse, and gelatinase B secreted by macrophages and polymorphonuclear leukocytes from these same species, and also by rabbit chondrocytes and endothelial cells. Crossed immunoblotting, antibody inhibition and deglycosylation studies indicated that gelatinase A and B are immunologically distinct gene products, although their substrate specificity profiles are identical. Gelatinase was most efficiently detected by the antibody in rabbit monocytes and connective tissue cells, whereas cells derived from human and pig gave poor immunostaining. It was concluded that these cross-reactions were not strong enough to permit immunolocalisation studies of tissues from these species, although mouse gelatinase stained well suggesting a strong cross-reaction (Murphy et al 1989b). In some experiments, to identify the cells synthesising gelatinase, an antibody to human 96kDa gelatinase B was used. The polyclonal antibody was raised in a sheep using as antigen the pig polymorphonuclear leukocyte gelatinase (Murphy et al 1989a). The antiserum recognised and specifically inhibited gelatinase B but not gelatinase A. On Western blotting the antiserum recognised gelatinase B but did not detect either purified recombinant human fibroblast collagenase or stromelysin.

Antiserum to rabbit stromelysin was raised in sheep by immunisation with purified stromelysin from rabbit bone culture media (Murphy et al 1986). The antiserum did not recognise purified rabbit bone collagenase or gelatinase by immunoblotting or immunoprecipitation but did recognise stromelysin from human fibroblast culture media and pig synovial culture media.
Antibody to tissue inhibitor of metalloproteinases-1 (TIMP-1) was developed by immunising sheep with a preparation of purified rabbit TIMP (Gavrilovic et al. 1987). The specificity of the antibody was investigated by immunoblotting. The antiserum recognised a single band of molecular weight 28kDa in crude rabbit bone, chondrocyte and endothelial cell culture medium, which ran in alignment with a band of purified rabbit bone TIMP-1 run on the same blot. The antiserum did not react with purified human or bovine TIMP-1 and therefore appears to be specific for rabbit TIMP-1.

Immunoglobulins from pooled normal sheep serum were prepared in the same way, NSS-IgG (Hembry et al. 1985) or normal sheep serum was obtained from Sigma, Poole, Dorset, U.K. and used with these antibodies as a control to determine non-specific binding of sheep IgG as well as non-specific reaction of the second antibody.

The second antibody was fluorescein isothiocyanate (FITC)-labelled rabbit F(ab')2 anti-sheep IgG (H+L chain specific) from Southern Biotechnology Associates Incorporated (Birmingham, Alabama, U.S.A.) which binds to all the primary antibodies employed allowing them to be visualised on a fluorescent microscope.

3.2.5 Histochemistry

Frozen sections 5μ thick were cut on a cryostat (Bright, Huntingdon, U.K.) and fixed for 30 minutes in 4 per cent paraformaldehyde (pH 7.35) in phosphate buffered saline (PBS composed of 8.5g NaCl, 3.21g Na₂HPO₄.2H₂O and 0.156g NaH₂PO₄.2H₂O per litre, adjusted to pH 7.35 with 5M HCl). After fixing, sections were washed in PBS. All washing stages comprised three changes of PBS each of 5 minutes. The tissue was then permeabilised in 0.1 per cent Triton X-100 for five minutes to allow subsequent penetration of antibodies into cells. After further washing, sections were treated for 10 minutes with 4-chloronaphthol, 2.8mM in methanol/PBS (1:5) containing 0.01 per cent hydrogen peroxide, to prevent the non-specific binding of FITC-
antibody by inflammatory cells (Johnston and Bienenstock 1974, Kingston and Pearson 1981). Sections were washed again and incubated at room temperature in a humid atmosphere for 30 minutes with the primary-antibodies, previously described, at 50 ug/ml in a blocking solution of 5% normal donkey serum (Sigma, Poole, Dorset, U.K.). Slides were washed to remove excess first antibody and incubated for 30 minutes with second antibody, rabbit anti-sheep-FITC Fab fragment IgG diluted 1/100 in a blocking solution of 2% rabbit albumin (Sigma, Poole, Dorset, U.K.) and 3% normal donkey serum. The sections were washed once more, stained for two minutes with methyl green (1mg/ml) as a nuclear counter-stain, and washed again. Dried slides were mounted in glycerol/PBS mounting fluid containing additives to reduce fading of fluorescence (Citifluor Ltd., Canterbury, U.K.). Sections were examined on the same day by epifluorescence using a Leitz Dialux 20 photomicroscope (Leica, Milton Keynes, U.K.) with standard wide and narrow-band filters. Sections were examined under low power with a rhodamine filter to map the layout of the tissue and then under higher powers with FITC standard wide and narrow band filters to determine the localisation of fluorescent antibody within the tissue. Slides incubated with normal sheep serum served as controls for the non-specific uptake of FITC antibody. Fluorescent photomicrographs were taken on Kodak Ektachrome 400 ASA film and Agfachrome 1000 RS.
CHAPTER 4

DEVELOPMENT OF MODELS

The rabbit was the most appropriate animal for the purpose of this study because physiologically and pharmacologically the rabbit colon is similar to the human colon (Percy et al 1989). Furthermore the antisera for immunolocalisation of MMPs and TIMP are only available to rabbit tissue and do not cross-react with other species.

The figures for this chapter are either inserted within the text or at the back of this chapter. Note that when stained with H&E, rabbit polymorphonuclear leukocytes stain with eosin, therefore appear “eosinophilic.”

4.1 Normal healing model

The normal healing model was adapted from the one used by Chowcat et al (1988). The colon was transected at the standard site (fig 4.1) and an anastomosis carried out but instead of an everted anastomosis an extra-mucosal anastomosis was performed as described in 3.2.1. Employing this technique a smooth anastomosis with a patent lumen was obtained with minimal inversion of the mucosa.

Of 17 rabbits prepared for this study 5 were found on sacrifice to have complications due to adhesive obstruction and partial or complete dehiscence of the suture line and were excluded from further study. From the remaining twelve rabbits, groups of 3 were killed at periods 12h, 24h, 3 days and 7 days after transection and anastomosis. A 2cm segment (with 1cm on either side of the suture line), and 2cm segments proximal and distal to it were excised for examination (fig 4.2a).
Figure 4.1 - Normal healing model

Diagram of blood supply to distal colon in rabbit showing location of transection and anastomosis used in normal healing experiments.
Figure 4.2 - Tissue segments taken for immunohistochemistry and H&E

a) Normal healing model

b) Obstruction model. Obstruction + anastomosis group
4.1.1 Sham operation

In this part of the study a sham operation was carried out in 4 rabbits. At laparotomy the distal colon was handled but not transected. Two animals were killed 24h after the operation and two after 3 days. In the region of the standard site a 3cm segment was resected for examination.

4.2 Intestinal obstruction model

To produce obstruction the method described by Tornqvist et al (1990a) in rats, placing a silastic ring (H.G.Wallace Limited, Essex, U.K.) around the colon was modified. In this model the ring was placed at the standard site in the distal colon (fig 4.3. a,b).

4.2.1 Development of the obstruction model

In 3 animals a silastic ring with an internal diameter of 7mm was placed around the colon and was left in place for 3 days before the rabbits were re-explored. At laparotomy there was minimal colonic distension above the obstruction and the distal bowel contained faeces. The colon encircled by the ring was resected and an anastomosis carried out. One rabbit died of peritonitis, two survived and were killed 3 days later. In these two animals H&E staining of the anastomotic segment showed a fibrin plug filling the gap between the cut ends of the bowel with dense acute and chronic inflammatory cell infiltrate. The mucosa was normal except for haemorrhage and cellular infiltration at the cut edges of this segment. Immunohistochemistry showed intra- and extra-cellular expression of MMPs and TIMP-1 restricted to the fibrous tissue, in the vicinity of the cut ends of the bowel. These findings were very similar to those observed in the animals killed 3 days after anastomosis in the normal healing model which is described in detail in 5.1.3.
Thus since the clinical and histological changes did not appear to differ from those in a non-obstructed animal a smaller ring of 3mm diameter was placed in three rabbits for 3 days. At re-exploration the bowel proximal to the ring was grossly distended and necrotic, therefore an anastomosis could not be carried out and these animals were discarded. The same sized ring was used in two other animals and left for 2 days only but these rabbits had to be discarded for the same reason. The time of obstruction was reduced to 24h again with a 3mm diameter ring. This caused dilatation without disruption of the bowel proximal to the ring although there was necrosis at the ring site with peritonitis in four rabbits which had to be discarded. Three rabbits survived and had their colon resected where the ring had been placed and an anastomosis carried out. They were sacrificed after 24h and the anastomotic segment excised for examination. H&E staining showed that the gap between the cut ends of the bowel wall was filled with fibrin which was heavily infiltrated with acute inflammatory cells. Differently from the normal healing model, oedema and inflammatory infiltration was not restricted to the suture line but extended proximally and distally throughout the submucosa, the muscle and the serosa. Another abnormality seen for the first time was loss of the epithelial mucosal surface along the whole length of one side of the anastomosis. Unlike the normal healing model, MMPs were immunolocalised extracellularly in the mucosa in the area where the epithelium was lost. Collagenase and stromelysin were the predominant enzymes although many cells containing gelatinase were seen in the submucosa. There was extracellular TIMP-1 in only a small area of the mucosa on the same side of the anastomosis.

It was apparent that the ring size required further modification in order to avoid necrosis at the ring site. A ring half the size of the bowel diameter left in place for 24h was tested. Using this method there was complete obstruction, with distension of the bowel proximal to the ring and collapse of the distal bowel but without tissue disruption at the ring site or of the distended bowel. Four rabbits, two each at 3 and 7 days after resection of the ring site and anastomosis were studied in this model. The resected ring
segment showed haemorrhage, oedema and an acute inflammatory cell infiltrate in all layers. At 3 days throughout the anastomotic segment there was oedema and haemorrhage in the submucosa and inflammatory cell infiltration of the fibrous tissue, the submucosa, the muscle and the serosa. In the mucosa there was extensive loss of the epithelial surface which was more marked on one side of the anastomosis than the other. The MMPs and TIMP-1 were localised extracellularly throughout the mucosa but only where the epithelium was disrupted. Intracellular gelatinase was seen in many cells in the submucosal layer. The proximal dilated segment of colon also showed extracellular collagenase, stromelysin and TIMP-1 in the mucosa and intracellular gelatinase in the submucosa. At 7 days the histological changes were confined to the fibrous tissue which had formed at the stitch line. This consisted principally of inflammatory cell infiltrate extending into the serosa. The MMPs and TIMP-1 were also restricted to the fibrous tissue and were present both intra- and extra-cellularly.

With the finding of a more widespread expression of MMPs and TIMP-1 in the anastomotic segment as well as in the proximal segment it was decided for subsequent studies on this model to taken tissue for H&E and immunohistochemistry at least 25cm proximal to the anastomosis which is the limit of the descending colon in the rabbit (fig 4.2.b).

4.2.2 The obstruction model

From these preliminary studies the technique developed to produce an obstruction was to place at the standard site a silastic ring for 24h. The ring was fashioned by cutting a length of silastic ribbon, half the bowel diameter measured at the standard site and stitched together around the colon (fig 4.3.b). The bowel diameter after 24h of obstruction was measured where the colon was maximally dilated. The bowel diameter was 8mm to 13mm (mean 10.4mm) and after obstruction for 24h ranged from 18mm to 24mm (mean 20.6mm).
The animals were re-explored after 24 hours to relieve the obstruction by releasing the ring or by resecting the ring site and carrying out an anastomosis. The animals were then sacrificed immediately or at different times varying from 12h to 7 days and colonic samples obtained from the ring/anastomotic site, the adjacent tissue distally and proximally at different levels (fig 4.2.b). Tissue was also sampled from the same sites 24h after placing a large ring with the same diameter of the colon, to determine whether silastic itself had any effect on the colonic tissue.

4.2.3 Ring resection and anastomosis

In 17 rabbits whose weights were 1850g to 2651g (mean 2212.1g), after 24h of obstruction their weight loss was between 39g and 105g (mean 69.3g). At laparotomy the findings were consistent with the established model already described. One or two blood vessels proximal and distal to the ring were ligated and a segment containing the ring with a cm margin of proximal bowel was resected and an anastomosis performed using the operative technique previously described (see 3.2.1) (fig 4.3). After anastomosis two animals were killed immediately. The other 15 had their abdomens closed and were allowed to recover. Seven rabbits died, 2 during anaesthesia, 3 on the third post-operative day with peritonitis and 2 after 5 days with intestinal obstruction. The remaining 8 animals were killed in groups of two at 12h, 24h, 3 days and 7 days, after resection and anastomosis; segments were taken from the anastomotic site, the adjacent 2cm segment distally and 2 consecutive 2cm segments proximally and a further 2cm segment 25cm proximal to the stitch line (fig 4.2.b).

4.2.4 Release of obstruction without anastomosis

In 11 rabbits ranging in weight from 2083g to 2885g (mean 2419.4g) after 24h of obstruction their weights were less by 70g to 118g (mean 100.4g). At re-exploration two rabbits had developed small bowel obstruction due to adhesions around the ring and were
discarded. The animals in groups of 3 were killed immediately, 24h and 3 days after the release of the silastic ring. Segments were taken from the sites already described in 4.2.3 but in view of the results which demonstrated extensive changes even in the segment 25cm from the suture line (described in 5.2.3), further segments were excised from the transverse (Trans) and ascending (Asc) colon (fig 4.4).

4.2.5 Placement of a large ring

In order to eliminate the possibility of an artefactual effect by silastic material, in 3 rabbits a ring of the same diameter as the bowel was fashioned and placed around the bowel at the standard site to allow contact without causing excessive compression on the bowel. At re-exploration 24h later, as expected, the bowel was not obstructed. The animals were sacrificed and colonic segments excised from the same sites as in 4.2.4.

4.3 Ischaemic model

The method described by Shandall et al (1985) was used to develop a model of ischaemia which had to satisfy clinical and histological criteria. These were black discoloration of the bowel wall, absence of peristalsis and histological evidence of tissue death, seen as loss of mucosal glandular structure, oedema and haemorrhage of the submucosa and muscle death demonstrated by loss or fragmentation of cell nuclei.

4.3.1 Development of the ischaemic model

In the distal colon at the standard site several vascular branches arising from the posterior mesenteric artery were ligated and divided for approximately 7cm as described by Shandall et al (1985). In order to determine the duration required for the bowel to become ischaemic 14 rabbits were studied, 3 were killed at each of 1, 3, 4 and 6 hours and 2 at 2 hours after induction of ischaemia. At the time of sacrifice the ischaemic
Figure 4.4 - Tissue segments taken for immunohistochemistry and H&E

a) Obstruction model. Obstruction only group

b) Ischaemic model

I - Resected ischaemic segment $\equiv 9\text{cm}$

II - Tissue taken after anastomosis
segment was resected and divided into approximately 3cm strips. 2cm segments were excised from the distal resection margin, two consecutive segments from the proximal margin, from a distance of 25cm from the suture line, from the transverse colon and ascending colon (fig 4.4). The blood flow was measured before and after devascularisation with a Periflux Laser Doppler Flowmeter PF 3 (PERIMED U.K. LTD, Suffolk, U. K.). A special probe was designed in which the rigid plastic holder of the standard probe was replaced by a 2cm circular disc of flexible latex (fig 4.5.a). This allowed its adherence to the bowel wall and measurements to be made despite the peristaltic movement of the colon (Special probe 315:74, PERIMED, Jarfalla, Sweden). The measurements were recorded on a potentiometric recorder calibrated so that 1 volt was equivalent to 100 perfusion units (PFU) on the laser doppler flowmeter. With the special probe there was no difficulty in attaching it to the intestinal surface and in obtaining a stable optical coupling without any tissue compression. Unusually high measurements due to peristaltic waves could therefore be discounted. Measurements were made in the ascending colon and distal colon (in the ischaemic region) before the induction of ischaemia, then in the ascending colon and at several points in the ischaemic segment just before killing. The tissue colour and peristaltic movements at the time the rabbits were killed were also noted.

In all animals killed 1 to 6 hours after devascularisation, the ischaemic bowel showed peristalsis and its colour varied from a faint bluish tinge after 1h of ischaemia to dark purple after 6h ischaemia (fig 4.5). At 6h there was gradation of colour from black at the centre of the ischaemic segment through lighter shades of purple to the normal pink colour at the proximal and distal boundaries of the ischaemic area (fig 4.5d).
Prior to ischaemia, laser doppler measurements in the ascending colon varied from 405 PFU to 721 PFU (mean 535 PFU) and in the distal colon ranged from 237 PFU to 671 PFU (mean 462 PFU). A wide range of results, as shown below, were measured in the ischaemic segment immediately before killing:

<table>
<thead>
<tr>
<th>Time</th>
<th>Animals</th>
<th>PFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>3</td>
<td>5, 8, 100</td>
</tr>
<tr>
<td>2 hours</td>
<td>2</td>
<td>13, 18</td>
</tr>
<tr>
<td>3 hours</td>
<td>3</td>
<td>5, 32, 88</td>
</tr>
<tr>
<td>4 hours</td>
<td>3</td>
<td>6, 31, 68</td>
</tr>
<tr>
<td>6 hours</td>
<td>3</td>
<td>10, 15, 48</td>
</tr>
</tbody>
</table>

Histological examination of the ischaemic segment showed no abnormalities after 1 and 2 hours of ischaemia (fig 4.6.a). At 3 hours there was only patchy loss of the epithelial surface with haemorrhage in a small area of the mucosa and the submucosa which was more extensive after 4 hours, but the muscle layer was spared (fig 4.6b and c). The rest of the ischaemic segment was normal. Six hours of ischaemia resulted in more widespread haemorrhage, now extending to the muscle layer, but the overall histological appearance was not dissimilar from that seen after 4h of ischaemia (fig 4.6.d).

No staining for MMPs and TIMP-1 was detected in the colonic tissue excised from either the ischaemic region or from any of the other segments except in one rabbit after 4 hours of ischaemia. In this animal extracellular collagenase, stromelysin, gelatinase and TIMP-1 were demonstrated in the mucosa; stromelysin and collagenase were more prominent and widespread. Intracellular gelatinase was also seen but only in the submucosa.
These changes were unconvincing and it appeared therefore that presumably unrecognisable collateral circulation had retained some vascularity of the supposedly ischaemic region. It was decided to ligate a few more vessels and extend the time of ischaemia to 12 hours as the ischaemic changes that occurred between 4 and 6 hours were considered only relatively minor.

Using these parameters three animals were tested. The lengths of the ischaemic segments were 9, 10 and 10.5 cm. It was observed at the time of killing that most of the ischaemic segment was black (fig 4.7), and only near the boundaries was there a transition to a dark purple colour. The normal bowel pink colour was seen beyond the area where the vessels had been ligated. There was no peristalsis in the ischaemic segment and no blood flow was occurring at the centre of the area as shown by zero PFU laser doppler measurements in all three rabbits. However, strong peristaltic waves were seen immediately outside the ischaemic region which also had a normal blood flow, with laser doppler readings of 468, 381, 434 PFU. Tissues were taken from the ischaemic segment (divided into A (proximal end), B (distal end) and C (centre) and from the same sites as described earlier in this section (fig 4.4 bl).

Histological examination of the bowel outside the ischaemic segment showed minor abnormalities only in the nearest segments, Prox0 and Distal. In both there was slight oedema and haemorrhage in the submucosa with polymorphonuclear cell and monocyte infiltration mainly in the muscle layer and in the serosa. These abnormalities were more marked in the Prox0 segment. Immunohistochemistry demonstrated only intracellular gelatinase in a few cells in the mucosa and submucosa in the Prox0 and in the Distal segments. None of the other segments showed any histological changes and did not stain for MMPs or TIMP-1.
Histological examination of the centre of the ischaemic segment (C) showed loss of the epithelial surface and destruction of the normal mucosal crypt pattern. The nuclei of the epithelial cells were misplaced and/or pycnotic and haemorrhage was so extensive that the lamina propria could not be identified. The submucosa showed loss of the normal connective tissue structure due to severe oedema and haemorrhage and there was polymorphonuclear leukocyte infiltration. There was destruction of the muscle striations with loss and fragmentation of the cell nuclei and considerable haemorrhage in this layer (fig 4.8). Towards both the proximal (A) (fig 4.9.a) and distal (B) (fig 4.9c) borders of the ischaemic segment more normal tissue could be identified although haemorrhage in all layers was still present.

The distribution of MMPs and TIMP-1 are shown in Table 5X and detailed in section 5.3.1.

The clinical and histological changes appeared compatible with the criteria laid out and the model was therefore based on the parameters employed to develop this degree of ischaemia.

4.3.2 The model of ischaemic bowel

Colonic ischaemia was induced in 9 rabbits, ranging in weight from 2100g to 2880g (mean 2402.1g), by ligating the small vessels arising from the posterior mesenteric artery, starting distally at a distance approximately 7cm from the peritoneal reflection and extending proximally for at least 9cm until a main branch of the posterior mesenteric artery was reached (fig 4.10). At re-exploration 12 hours later the weight loss ranged from 48g to 107g (mean 77.5g). The ischaemic segment was resected dividing the bowel at the level of the proximal and the distal vascular ligatures where the colon was of a normal pink colour. The occurrence of bleeding at the cut edges of bowel was noted. An anastomosis was performed as previously described (see 3.2.1). The rabbits
Figure 4.10

Diagram showing the blood vessels tied to produce an ischaemic segment in the distal colon.
were allowed to recover and were sacrificed in groups of 3 after 12h and 24h and two after 3 days (the third died on second day) and segments were resected from the anastomotic site, the adjacent 2cm distally and 2 consecutive 2cm segments proximally, further 2cm segments were excised from a position 25cm from the suture line, from the transverse colon and from the ascending colon.
Figure 4.3 - Obstruction model

(a) Diagram of blood supply to distal colon in rabbit showing site of ring and resection used in obstruction experiments.

(b) Photograph showing site of the ring immediately after placing it. After 24h the vessels either side of the ring were tied and a segment containing the ring resected.

px - proximal bowel  d - distal bowel  pma - posterior mesenteric artery
Figure 4.3. Obstruction model
c)

Figure showing the distal colon 24 h after placement of the silastic ring. The proximal bowel is dilated more than 2x the normal diameter and the distal bowel is collapsed. The darker colour in the proximal bowel is due to liquid faeces in the lumen.  p-proximal bowel  d-distal bowel  r-ring
Figures show the resected segment containing the silastic ring. The proximal side (px) is larger than the distal (d) and is slightly darker in colour.
Fig 4.3 - Obstruction model

Figure shows anastomosis 24 h after resection of the ring and anastomosis. The proximal bowel is darker than the distal one. Note the small inversion of the mucosa.
Figure 4.5 Ischaemic model
Figures show the distal colon of the rabbit at the site of ischaemia.

a)

Normal blood supply showing the posterior mesenteric artery (arrow) and the Laser Doppler probe (arrow head). Small arrows indicate the vessels to be tied.

b)

The distal bowel after 2 h of ischaemia. Only a slight change in colour is noted compared to fig. 4.5.a. pma-posterior mesenteric artery
Figure 4.5. Ischaemic model. Figures show the distal bowel of the rabbit at the site of ischaemia.

c) Macroscopic view of the distal colon after 4 h of ischaemia, the ischaemic segment is becoming progressive darker; compare to 4.5.a, b.

d) Macroscopically after 6 h the ischaemic segment appears necrotic.
After 2 h of ischaemia, except for slight haemorrhage in the serosa, the histology was normal. Note the preserved epithelium and the normal submucosa and muscle. (See fig 4.1 for normal colon). m-mucosa sm-submucosa mu-muscle s-serosa

View of the ischaemic segment 4 h after tying the vessels. Slight haemorrhage can be seen in the mucosa in areas where the epithelial border has been lost. The abnormalities at this time were confined to the mucosa. m-mucosa sm-submucosa mu-muscle.
This section from the centre of the ischaemic segment after 4 h of ischaemia still has an epithelial border but haemorrhage of the mucosa can be seen. The submucosa and muscle are normal.

m-mucosa sm-submucosa mu-muscle

Abnormalities seen after 6 h of ischaemia were superficial ulceration and slight haemorrhage in the mucosa. Haemorrhage and oedema of the submucosa and muscle were also present.

m-mucosa sm-submucosa mu-muscle
The ischaemic segment after 12 h appears totally black and therefore necrotic.
Both figures show the histological lesions in the necrotic bowel after 12 h of ischaemia. In a) the haemorrhage extends to all layers and is so extensive that is difficult to recognise the bowel structure. In the mucosa the glands are hard to distinguish being apparently much further apart than usual. The submucosa appears as a mass of red blood cells. Haemorrhage also distorts the normal muscular architecture. In b) there is less haemorrhage but it still dissociates muscle bundles and mucosal glands.
m-mucosa  sm-submucosa  mu-muscle
Figure 4.8. Ischaemic model
Figures show the centre of the ischaemic segment

c) Original magnification x 200

![Image of figure c)

Original magnification x 200]

Both figures are magnifications of fig 4.8.b showing the muscle layer. Muscle fibres are dissociated by oedema and haemorrhage and polymorphonuclear cells (arrows) can be seen infiltrating the muscle bundles.


d) Original magnification x 400

![Image of figure d)

Original magnification x 400]
a) Original magnification x 40

Near the proximal border of the ischaemic segment (px) only slight haemorrhage in the mucosa and muscle is seen. Whereas at the distal (d) end, near segment C, there are epithelial lesions of the mucosa and a more extensive haemorrhage in the mucosa, submucosa and muscle. m-mucosa  sm-submucosa  mu-muscle

b) Original magnification x 25

In the region nearest to the centre of the ischaemic segment necrotic tissue occurs, demonstrated by extensive mucosal crypt disruption and heavy haemorrhage that extends to the submucosa and muscle, with complete loss of the normal bowel architecture. m-mucosa  sm-submucosa  mu-muscle
Near the distal border of the ischaemic segment, except for minor vascular congestion, the bowel wall is normal.
m-mucosa  mm-muscularis mucosae  sm-submucosa

Proximal to the centre a necrotic bowel is seen with complete loss of the normal bowel wall architecture. The more extensive damage and haemorrhage is seen in the mucosa but also haemorrhage can be seen in the muscle layer. m-mucosa  sm-submucosa  mu-muscle
CHAPTER 5

RESULTS

The histological changes and the distribution of the MMPs and TIMP-1 although consistent were not uniform in all the animals, therefore description of the findings was based on the overall pattern observed.

The figures are at the end of the chapter. Rabbit polymorphonuclear leukocytes stain “eosinophilic” with H&E.

5.1 Normal healing model

Sixteen rabbits, 12 after transection and anastomosis and 4 after a sham operation were studied (3 each at 12h, 24h, 3 days and 7 days with anastomosis and 2 each at 24h and 3 days after a sham operation). At laparotomy the anastomoses looked intact and except for flimsy adhesions to the bladder and small bowel in animals killed on the seventh post-operative day the findings were unremarkable.

5.1.2 Tissue from sham operations

Tissues of segments resected from the standard site in the animals that had undergone a sham operation showed no histological changes or staining for any of the MMPs or TIMP-1 (fig 5.1).

5.1.3 Tissue from anastomotic region

Tissue examined was from the anastomotic segment and from the colon immediately proximal and distal to the anastomosis.
The histological appearance of the segments proximal and distal to the anastomoses was normal and there was no demonstrable staining to any of the MMPs or TIMP-1 at any time after transection and anastomosis. The histological and immunohistochemical changes described here were confined entirely to the anastomotic segment.

12 hours post-anastomosis

A fibrin clot infiltrated with acute inflammatory cells filled the gap between the cut ends of the bowel. There was oedema of the submucosa and the muscle layer as well as haemorrhage in all the layers of the bowel wall (fig 5.2.a,c,d).

MMPs were confined to the region of the suture line, in the mucosa, submucosa and muscle, concentrated at the edges of the divided bowel (fig 5.2.b, Table 5.1). In the mucosa both intra- and extra-cellular enzymes were seen but there was much less stromelysin than collagenase and gelatinase (fig 5.2.b, e,f,g,h). In the submucosa intracellular collagenase and gelatinase were present whereas in the muscle layer only collagenase both extra- and intra-cellular was evident (Table 5 - 1).

TIMP-1 was seen intra- and extra-cellularly in the mucosa in the same area as the MMPs but not as widely distributed. In the submucosa and the muscle layer a few cells stained for TIMP-1 but there was no evidence of extracellular TIMP-1 (fig 5.2.b). No MMPs or TIMP-1 were seen in the serosa (Table 5.1).
Table 5.I - Normal healing model  
Distribution of MMPs and TIMP-1 after anastomosis

| Tissue segment | Time  | Mucosa | | Submucosa/fibrous tissue | | Muscle |
|----------------|-------|--------| | Extra | Intra | Extra | Intra |
| Distal         |       |        | |        | |        | |
| Anas           | 12 h  | □ □ □ □ | | □ □ □ □ | | □ □ □ □ |
|                | 24 h  | □ □ □ □ | | □ □ □ □ | | □ □ □ □ |
|                | 3 days| □ □ □ □ | | □ □ □ □ | | □ □ □ □ |
|                | 7 days| □ □ □ □ | | □ □ □ □ | | □ □ □ □ |
| Prox0          |       |        | |        | |        | |

Collagenase - □ □ □ □  Gelatinase - □ □ □ □  Stromelysin - □ □ □ □  TIMP - □ □ □ □  Squares - extracellular  Circles - intracellular

Each result represents the pattern seen in 3 rabbits at each time point
24 hours post-anastomosis

The overall histological appearance was similar to that observed at 12h (fig 5.3.a). The MMPs and TIMP-1 were localised only at the suture line of the anastomotic segment (fig 5.3.b, Table 5.1). In the mucosa MMPs spread further away from the cut ends than at 12 hours and also into the submucosa; only extracellular collagenase was present whereas both intra- and extra-cellular stromelysin and gelatinase were seen. In the submucosa MMP staining was more evident at the bowel ends, continuous with the mucosal layer. Both intra- and extra-cellular collagenase and gelatinase were present and were more widespread than stromelysin which was only seen extracellularly. Only intracellular gelatinase and extracellular collagenase were detected in the muscle layer and no MMPs were seen in the serosa (Table 5.1).

TIMP-1 was present in all layers, extracellularly in the mucosa and spreading in the submucosa within the same area as collagenase but not as extensively. In the muscle it was in the proximity of the area stained with collagenase and gelatinase (fig 5.3.b, Table 5.1).

3 days post-anastomosis

The fibrin plug was more dense and heavily infiltrated with acute and chronic inflammatory cells and fibroblasts occupying the gap between the bowel ends. This extended into the deeper layers so that the individual layers were unrecognisable (fig 5.4.a). A few epithelial cells and muscle fragments were seen in the fibrin plug, undergoing degradation. The serosal surface was sealed with a layer of connective tissue.

As in the earlier periods MMPs and TIMP-1 were confined to the anastomotic line within the fibrin plug (fig 5.4.b, Table 5.1). Extra- and intra-cellular collagenase,
gelatinase and stromelysin were not only present at the margin of the cut edges of the mucosa but also in the fibrous plug; stromelysin now was more widespread than collagenase and gelatinase (fig 5.4.b).

The distribution of the enzymes was wider and the staining heavier in the fibrous plug. TIMP-1 was present both extra- and intra-cellularly in the same locations as the MMPs although the fluorescence was not as bright or as widespread (fig 5.4.b, Table 5.1).

7 days post-anastomosis

The continuity of the mucosa was almost restored by epithelial migration. In the deeper layers, the fibrous tissue had matured and contained some buried mucosal crypts and muscle cells as well as numerous inflammatory cells. A connective tissue layer sealed the serosal surface (fig 5.5.a,c,d).

The most dense fluorescence was in the fibrous tissue and was entirely intracellular (fig 5.5.b, Table 5.1). Gelatinase although intracellular in cell clusters was the dominant enzyme in the fibrous tissue (fig 5.5.e). Collagenase and stromelysin were also present (fig 5.5.f). Extracellular gelatinase and stromelysin were only seen in the mucosa adjacent to the fibrous tissue (fig 5.5.g) as well as extracellular collagenase although was not as widespread. No staining for the MMPs was seen in the muscle. The newly formed serosal layer, for the first time showed MMP staining throughout the anastomotic segment mainly intracellular stromelysin. Intracellular gelatinase was also present localised in clusters of cells between the deeper fibrous tissue and the overlying serosal connective tissue. Occasional cells stained for collagenase (Table 5.1).

TIMP-1 was only present intracellularly in the fibrous tissue co-localising with the MMPs (fig 5.5.b, Table 5.1)
5.1.4 Summary of results in the normal healing model

The histological appearance exhibited a normal evolution of anastomotic healing. In the first 24 hours a fibrin plug infiltrated by inflammatory cells with oedema and haemorrhage was seen between the cut edges of the bowel. This developed to a fibrous tissue with dense infiltration of inflammatory cells and fibroblasts by 3 days. A mature fibrous tissue was formed at 7 days between the bowel ends. Epithelial migration restored the mucosal continuity and the serosa was sealed by a connective tissue layer. There were no histological abnormalities proximal of distal to the suture line.

The immunohistochemistry demonstrated that MMPs and TIMP-1 were localised to the suture line from 12h to 7 days after anastomosis. At 12h extracellular MMPs, mainly collagenase were seen predominantly in the mucosa. At 24h extracellular MMPs were equally present in the mucosa but more was seen in the submucosa and in the fibrin plug. By the third day more intra- than extra-cellular enzymes were in the fibrous plug mainly stromelysin. At 7 days, intracellular gelatinase was dominant in the fibrous tissue and intracellular stromelysin in the serosa. During the whole period TIMP-1 was present, but not as widely distributed as the MMPs, co-localised with the enzymes and by the seventh day none was seen in the mucosa or the serosa and was confined to the fibrous tissue. Neither the MMPs nor TIMP-1 were seen in the proximal or distal segments.

5.2 Obstruction model

A total of 19 animals were studied, 3 each at 0h, 24h and 3 days after releasing the obstruction without resection and 2 each at 0h, 12h, 24h, 3 days and 7 days after resection and anastomosis, 3 rabbits were also studied 24h after placing a large silastic ring round the distal colon as a control. At laparotomy the anastomoses appeared intact.
Few adhesions to the bladder or small bowel were seen in the animals killed at 3 and 7 days.

5.2.1 Tissue from the obstruction site (ring site)

The ring site was examined at every resection. There was patchy loss of the epithelial border, mild inflammatory reaction in all layers with oedema and haemorrhage in the submucosa and the muscle layers. The only MMP present was intracellular gelatinase, none of the other enzymes or TIMP-1 were seen.

5.2.2 Tissues from animals with anastomosis

Tissue was examined from the anastomotic segment and from the colon distally and proximally in two serial segments (Prox0 and Prox1) and from the descending colon 25cm from the suture line (Prox2).

0 hour post-anastomosis

In the two animals killed immediately after anastomosis only mild oedema and chronic inflammatory cell infiltrate were seen in the submucosa of the distal segment whereas in the anastomotic segment (only proximal to the stitch line) and in Prox0, Prox1 and Prox2 there was loss of the epithelial border of the mucosa with oedema and inflammatory cell infiltration of the submucosa and the muscle layer.

Collagenase and to a lesser degree stromelysin were widespread extracellularly in the mucosa of Anas, Prox0 and Prox1 (Table 5.II). Extracellular collagenase was also present in Prox2. Gelatinase was more widespread intracellularly than collagenase and stromelysin not only in the mucosa and submucosa but also in the muscle layer in these segments, as well as in Prox2.
Table 5.II - Obstruction model
Distribution of MMPs and TIMP-1 immediately after formation of an anastomosis following an obstruction

<table>
<thead>
<tr>
<th>Tissue segment</th>
<th>Rabbit n°</th>
<th>Mucosa</th>
<th>Submucosa</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Extra</td>
<td>Extra</td>
<td>Extra</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intra</td>
<td>Intra</td>
<td>Intra</td>
</tr>
<tr>
<td>Distal</td>
<td>D49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anas</td>
<td>D49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox0</td>
<td>D49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox1</td>
<td>D49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox2</td>
<td>D49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D52</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Collagenase - Green, Gelatinase - Red, Stromelysin - Blue, TIMP - Light Blue, Squares - extracellular, Circles - intracellular
Extracellular TIMP-1 was scantily distributed in the mucosa of Anas, Prox0 and Prox1 and only intracellular TIMP-1 was present in a few cells in the submucosa of Prox0 and Prox1 as well as in the mucosa of the Distal segment (Table 5.II).

**12 hours post-anastomosis**

In the anastomotic segment the gap between the cut ends of the bowel was filled with a fibrin clot, oedema and fragments of mucosa and muscle (fig 5.6.a). Although early, the inflammatory reaction was intense with significant increase in cellularity in the fibrin plug as well as in the mucosa and in the deeper layers throughout this segment (fig 5.6 c, d). The main inflammatory cells were the polymorphonuclear leukocytes and macrophages. There was loss of the epithelial border of the mucosa with oedema, haemorrhage and inflammatory infiltrate, immediately proximal to the stitch line (fig 5.6.c).

In the distal segment there was epithelial damage only in small areas with slight oedema and mainly monocyte infiltrate. A few haemorrhagic areas and early inflammatory infiltrate consisting of macrophages, monocytes and polymorphonuclear leukocytes were the main features in the submucosa and the muscle layers. Prox0 showed heavier infiltration of inflammatory cells predominantly monocytes in the mucosa and polymorphonuclear leukocytes in the submucosa, the muscle layer and the serosa with thickening of the muscle and submucosal layers by haemorrhage and oedema. The histological changes were similar in Prox1 and Prox2 (fig 5.7 a, b). Patchy loss of the epithelial border was seen in the mucosa of all proximal segments, more extensively in Prox2 and Prox0 segments.

In the anastomotic segment all the enzymes and TIMP-1 were present, extracellularly, in the mucosa (fig 5.6.b, Table 5.III) although some collagenase and gelatinase were also seen in the submucosa. The most widely and evenly distributed,
<table>
<thead>
<tr>
<th>Tissue segment</th>
<th>Rabbit n°</th>
<th>Mucosa</th>
<th>Submucosa/fibrin plug</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Extra</td>
<td>Extra</td>
<td>Intra</td>
</tr>
<tr>
<td>Distal</td>
<td>D26</td>
<td>![Green Square]</td>
<td>![Red Square]</td>
<td>![Red Square]</td>
</tr>
<tr>
<td></td>
<td>D32</td>
<td>![Green Square]</td>
<td>![Red Square]</td>
<td>![Red Square]</td>
</tr>
<tr>
<td>Anas</td>
<td>D26</td>
<td>![Green Square]</td>
<td>![Red Square]</td>
<td>![Red Square]</td>
</tr>
<tr>
<td></td>
<td>D32</td>
<td>![Green Square]</td>
<td>![Red Square]</td>
<td>![Red Square]</td>
</tr>
<tr>
<td>Prox0</td>
<td>D26</td>
<td>![Green Square]</td>
<td>![Red Square]</td>
<td>![Red Square]</td>
</tr>
<tr>
<td></td>
<td>D32</td>
<td>![Green Square]</td>
<td>![Red Square]</td>
<td>![Red Square]</td>
</tr>
<tr>
<td>Prox1</td>
<td>D26</td>
<td>![Green Square]</td>
<td>![Red Square]</td>
<td>![Red Square]</td>
</tr>
<tr>
<td></td>
<td>D32</td>
<td>![Green Square]</td>
<td>![Red Square]</td>
<td>![Red Square]</td>
</tr>
<tr>
<td>Prox2</td>
<td>D26</td>
<td>![Green Square]</td>
<td>![Red Square]</td>
<td>![Red Square]</td>
</tr>
<tr>
<td></td>
<td>D32</td>
<td>![Green Square]</td>
<td>![Red Square]</td>
<td>![Red Square]</td>
</tr>
</tbody>
</table>

along the entire length of the mucosa and the submucosa, was collagenase. Stromelysin, gelatinase and TIMP-1 were unevenly distributed along this segment and except for stromelysin were restricted to the mucosa (fig 5.6.e,f,g,h). Throughout this segment, in all layers, especially in the submucosa, intracellular gelatinase was consistently present (fig 5.6.i,j,k and Table 5.III).

All extracellular enzymes, mainly collagenase, were expressed but not as widely or evenly distributed in the mucosa of the distal and proximal segments as in the anastomotic segment. Intracellular gelatinase was present in the mucosa, submucosa and muscle layer of all segments (Table 5.III).

TIMP-1 distribution was constant, either extracellularly or intracellularly in the mucosa and the submucosa of the anastomotic segment and the Distal and Prox0 segments, but was never present in the muscle layer or in the other proximal segments examined (Table 5.III).

24 hours post-anastomosis

In the anastomosis area a large gap between the cut edges of the bowel wall was filled with a large fibrin clot and remnants of mucosal glands and muscle fragments, numerous fibroblasts, mononuclear cells and polymorphonuclear leukocytes (fig 5.8.a, c). The submucosa was thickened with oedema and haemorrhage. The infiltration of inflammatory cells into the mucosa was larger than at 12 hours, especially around the sutures (fig 5.8. c) and were mainly mononuclear cells. In the submucosa, the muscle layer and the serosa there were as many polymorphonuclear leukocytes as mononuclear cells. Haemorrhage was seen between the muscle fibres.
### Table 5.IV - Obstruction model

Distribution of MMPs and TIMP-1 24 h after resection and anastomosis following an obstruction

<table>
<thead>
<tr>
<th>Tissue segment</th>
<th>Rabbit n°</th>
<th>Mucosa</th>
<th>Submucosa/fibrin plug</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Extra</td>
<td>Extra</td>
<td>Extra</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intra</td>
<td>Intra</td>
<td>Intra</td>
</tr>
<tr>
<td>Distal</td>
<td>D36</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>D39</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Anas</td>
<td>D36</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>D39</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Prox0</td>
<td>D36</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>D39</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Prox1</td>
<td>D36</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>D39</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Prox2</td>
<td>D36</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td>D39</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

**Legend:**
- Collagenase - □
- Gelatinase - □
- Stromelysin - □
- TIMP - □
- Squares - extracellular
- Circles - intracellular
In the other segments the infiltration of the mucosa by polymorphonuclear leukocytes and mononuclear cells was more pronounced than at 12 hours, and was far less in the deeper layers of the wall. Oedema and haemorrhage in these layers had diminished during this period.

All the MMPs and TIMP-1 were seen extracellularly in the anastomotic segment in the vicinity of the suture line and distributed unevenly throughout the mucosa (fig 5.8.b, Table 5.IV). Collagenase was more widely distributed than the other enzymes, and was the sole enzyme present in the submucosa. Only intracellular gelatinase was seen in the deeper layers of this segment. TIMP-1 fluorescence was not as bright or as extensive as the MMPs (Table 5.IV).

In the other segments extracellular collagenase was found in the mucosa of the Distal, Prox0 and Prox1 segments, as dense but not as even in its distribution as in the anastomotic segment and by the Prox2 segment collagenase fluorescence was not as bright or as widely distributed. Extracellular gelatinase and stromelysin were detected in the mucosa of all segments, but not with the same intensity as collagenase. Intracellular gelatinase was the sole enzyme present in all segments, particularly in the Distal and Prox0 mainly in the submucosa and the muscle layer (Table 5.IV).

TIMP-1 was seen in the anastomotic and the Distal segments but not in the proximal segments, except Prox 2 extracellularly and mainly in the mucosa but not with the same intensity as the MMPs (Table 5.IV).

3 days post-anastomosis

In the anastomotic segment there was an increase in the number of inflammatory cells and fibroblasts confined to the fibrous plug with minimal inflammatory reaction in the remaining segments (fig 5.9.a).
Table 5.V - Obstruction model
Distribution of MMPs and TIMP-1 3 days after resection and anastomosis following an obstruction

<table>
<thead>
<tr>
<th>Tissue segment</th>
<th>Rabbit n°</th>
<th>Mucosa</th>
<th>Submucosa/fibrous tissue</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Extra</td>
<td>Extra/fibrous tissue</td>
<td>Extra</td>
</tr>
<tr>
<td>Distal</td>
<td>D27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anas</td>
<td>D27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox0</td>
<td>D27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox1</td>
<td>D27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox2</td>
<td>D27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D37</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Collagenase - □  Gelatinase - □  Stromelysin - □  TIMP - □  Squares - extracellular  Circles - intracellular
The overall picture in the anastomotic segment was very different from 12 hours and 24 hours post-anastomosis. All the enzymes and TIMP-1 were confined to the suture line near the cut edges of the bowel, not only in mucosa, but also in the fibrous tissue plug (fig 5.9.b, Table 5.V). Extracellular collagenase was the dominant enzyme in the mucosa, the fibrous tissue and the muscle layer (fig 5.9.c). Extracellular gelatinase (fig 5.9.e) and stromelysin (fig 5.9.f) were seen in the mucosa and the fibrous tissue. Gelatinase was more widespread than stromelysin. TIMP-1 was seen at the same site but with less intensity of staining (fig 5.9.d). Intracellular gelatinase was mostly concentrated around the suture line and mainly in the submucosa and muscles.

The distribution of the enzymes had also changed in the other segments. Extracellular collagenase was confined to the distal and Prox 2 segments and with a lesser density extracellular gelatinase was seen in all segments except Prox1 (Table V). Extracellular stromelysin was the most widespread MMPs at this time and was solely present in Prox 1 segment. Intracellular gelatinase was present in all layers of all segments, except the Distal, and was most dense in the anastomotic segment (Table V).

TIMP-1 was positive extracellularly in the mucosa of all segments, except Prox 0 but not in the other layers (Table 5.V).

7 days post-anastomosis

The continuity of the mucosa was almost restored by one week (fig 5.10.a). In the deeper layers a maturing fibrous tissue had formed, which still contained inflammatory cells and some buried mucosal crypts and muscle fibres. The serosa was sealed by a layer of connective tissue. Outside the region of the fibrous tissue and in all others segments the structure of the bowel wall had returned to normality.
Table 5.VI - Obstruction model

Distribution of MMPs and TIMP-1 7 days after formation of an anastomosis following an obstruction

<table>
<thead>
<tr>
<th>Tissue segment</th>
<th>Rabbit n°</th>
<th>Mucosa</th>
<th>Submucosa/fibrous tissue</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Extra</td>
<td>Extra</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intra</td>
<td>Intra</td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td>D33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anas</td>
<td>D33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox0</td>
<td>D33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox1</td>
<td>D33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox2</td>
<td>D33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D40</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Collagenase -  Green, Gelatinase -  Red, Stromelysin -  Blue, TIMP -  Yellow, Squares - extracellular, Circles - intracellular
MMPs and TIMP-1 were only seen in the anastomotic segment but not in the other segments (fig 5.10.b, Table 5.VI). In the mucosa only intra- and extra-cellular stromelysin were present near the cut edges of the bowel. Extracellular collagenase, stromelysin, gelatinase and TIMP-1 were seen in the fibrous plug between the cut bowel ends. Collagenase exhibited the brightest fluorescence and was the most widely distributed MMPs in the fibrous tissue. Intracellular enzymes and TIMP-1 also occurred in the deep fibrous tissue near the serosa (Table 5.VI).

5.2.3 Placement of a large ring

A large ring the same size as the diameter of the bowel had no effect on the bowel histology. The sections showed a normal mucosal structure with no loss of the epithelial border or crypt architecture nor was there any oedema of the submucosa.

None of the MMPs or TIMP-1 were ever localised in sections of the colon from the site of the large ring.

5.2.4 Summary of results in the group of obstruction and anastomosis.

Epithelial and crypt disruption were seen discontinuously in the mucosa accompanied by oedema, haemorrhage and a large acute cell inflammatory infiltrate, in the submucosa, muscle and serosa from the distal to the Prox2 segment. The histological picture 12h post-operation was the same but unlike the group without anastomosis at 24h post-anastomosis there was still extensive damage to the tissue (see 5.2.5). The inflammatory infiltrate was more extensive than in the 12h animals and contained cells associated with chronic inflammation. At three and seven days histological alterations were seen only in the anastomotic segment mainly in the region of fibrous tissue.
The MMPs and TIMP-1 were immunolocalised extracellularly, mainly collagenase, in the mucosa in all the segments from Distal to Prox2 in the animals sacrificed immediately after anastomosis and unlike the group of obstruction only, the expression of the enzymes and TIMP-1 persisted in the animals killed at 24h. At 3 days there was still extracellular enzymes in the mucosa although less than at 24h. At 7 days only in the anastomotic segment were extracellular enzymes seen. The most widespread enzymes were collagenase and stromelysin.

Intracellular enzymes were observed in all segments mainly in the submucosa at 0h and 12h but at 24h and 3 days only intracellular gelatinase was present. At 7 days there were only few cells secreting stromelysin and gelatinase in the fibrous tissue of the anastomotic segment.

There were no histological abnormalities in the colon in rabbits having a large ring, nor were any MMPs or TIMP-1 seen.

5.2.5 Tissues from animals without anastomoses

Groups of 3 animals were studied immediately, 24h and 3 days after the release of the silastic ring. Tissue was taken from the ring site, immediately distally and proximally in two serial segments (Prox0 and Prox1), and from the descending colon 25cm from the suture line (Prox2). Because in the study of obstruction and anastomosis (5.2.4) enzymes were still present in Prox 2, in this study segments of tissue were also taken from the transverse (Trans) and ascending (Asc) colon.
Immediately after releasing the obstructing ring:

The segment distal to the ring showed only minor changes consisting of slight oedema in the submucosa and infiltration of acute inflammatory cells in the serosa. Proximal to the obstruction extensive mucosal damage occurred in Prox0 (fig 5.11.a,c,d) and Prox1 (5.12.a,c,d) segments. There was loss of the epithelial border with destruction of the crypts, and acute inflammatory cell infiltrate, oedema and haemorrhage in all the layers of the bowel wall. Minimal changes were seen in Prox2. The segments taken from the transverse colon, with the exception of one rabbit which had oedema of the submucosa, and from the ascending colon were normal.

In the Distal, Prox0 and Prox1, both extra- and intra-cellular MMPs were seen in the mucosa, mainly extracellular collagenase and intracellular gelatinase (Table VII). Also widely distributed was extracellular stromelysin but extracellular gelatinase was not as abundant. In the deeper layers only intracellular MMPs were present. In more proximal segments (Prox2, Trans and Asc) all layers stained for intra- but not extracellular MMPs, which appeared to be less prominent in the muscle layer and in the ascending colon. Stromelysin was absent in the muscle layer from Prox1 to Asc.

Mucosal extracellular TIMP-1 was observed only in Prox0 and Prox1 segments but was less widespread than the MMPs. Intracellular TIMP-1 was scantily distributed in all layers and in all segments, except the ascending colon (Table 5.VII).
Table 5.VII - Obstruction model
Distribution of MMPs and TIMP-1 in the animals killed after 24 h of obstruction only

<table>
<thead>
<tr>
<th>Tissue segment</th>
<th>Rabbit n°</th>
<th>Mucosa Extra</th>
<th>Mucosa Intra</th>
<th>Submucosa Extra</th>
<th>Submucosa Intra</th>
<th>Muscle Extra</th>
<th>Muscle Intra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distal</td>
<td>D44</td>
<td>D44</td>
<td>D47</td>
<td>D51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Prox0</td>
<td>D44</td>
<td>D44</td>
<td>D47</td>
<td>D51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td></td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Prox1</td>
<td>D44</td>
<td>D44</td>
<td>D47</td>
<td>D51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td></td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Prox2</td>
<td>D44</td>
<td>D44</td>
<td>D47</td>
<td>D51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td></td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Trans</td>
<td>D44</td>
<td>D44</td>
<td>D47</td>
<td>D51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td></td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Asc</td>
<td>D44</td>
<td>D44</td>
<td>D47</td>
<td>D51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td></td>
<td></td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

24 hour after the release of the obstructing ring

In all animals the histological changes were far less severe than those seen immediately after releasing the obstruction. There was only slight oedema, haemorrhage and acute and chronic inflammatory cell infiltrate in the submucosa and in the muscle layer in the Distal, Prox0, Prox1 and Prox2. Epithelial disruption of the mucosa was only seen in one animal in Prox0 and was patchy. The more proximal segments did not show any histological change.

In the mucosa, in contrast with the previous observation no extracellular MMPs or TIMP-1 were demonstrated in any segment in all animals (Table VIII). Only intracellular MMPs were present scattered throughout the different layers of the bowel wall mainly of Distal, Prox0, Prox1 and Prox2, and much less intense in Trans and Asc segments. Intracellular gelatinase was the predominant enzyme. TIMP-1 was also confined to the same areas (Table 5.VIII).

3 days after release of the obstructing ring

By this time there was unremarkable histological damage and in one animal complete restoration to normality had occurred. The abnormalities consisted of mild infiltration of chronic inflammatory cells in the submucosa of all segments and slight oedema in Prox0 and Prox1 segments.

The expression of MMPs and TIMP-1 reflected the structural return to normality as only a few cells mainly in the submucosa in Prox0, Prox1 and Prox2 segments showed intracellular gelatinase and negligible stromelysin and TIMP-1 (Table 5.IX).
## Table 5.VIII - Obstruction model

Distribution of MMPs and TIMP-1 in the animals killed 24 h after releasing the obstruction

<table>
<thead>
<tr>
<th>Tissue segment</th>
<th>Rabbit n°</th>
<th>Mucosa</th>
<th>Submucosa</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Extrá</td>
<td>Intrá</td>
<td>Extrá</td>
</tr>
<tr>
<td>Distal</td>
<td>D41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox0</td>
<td>D41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox1</td>
<td>D41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox2</td>
<td>D41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans</td>
<td>D41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asc</td>
<td>D41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D50</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.IX - Obstruction model

Distribution of MMPs and TIMP-1 in the animals killed 3 days after releasing the obstruction

<table>
<thead>
<tr>
<th>Tissue segment</th>
<th>Rabbit n°</th>
<th>Mucosa Extra</th>
<th>Mucosa Intra</th>
<th>Submucosa Extra</th>
<th>Submucosa Intra</th>
<th>Muscle Extra</th>
<th>Muscle Intra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distal</td>
<td>D46, D48, D53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox0</td>
<td>D46, D48, D53</td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox1</td>
<td>D46, D48, D53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox2</td>
<td>D46, D48, D53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans</td>
<td>D46, D48, D53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asc</td>
<td>D46, D48, D53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Collagenase - ▶️ Gelatinase - ▶️ Stromelysin - ▶️ TIMP - ▶️ Squares - extracellular ▶️ Circles - intracellular
5.2.6 Summary of results in the group of obstruction without anastomosis

Animals killed after 24h of obstruction showed histological damage immediately proximal and distal to the ring site. In the mucosa there was loss of the epithelial border, as well as haemorrhage and oedema with inflammatory cell infiltrate involving the other layers of the bowel wall. In the rest of the colon, there was only some oedema and inflammatory cell infiltrate. The histological changes were less marked 24h after releasing the obstruction and had virtually resolved by 3 days.

Initially at the release of obstruction there was expression of extracellular collagenase, stromelysin, and gelatinase restricted to mucosa of the segments immediately above and below with TIMP-1 in the same area. Intracellular MMPs and TIMP-1 were present in all layers and spread along the entire colon. After 24h only intracellular MMPs, predominantly gelatinase, and TIMP-1 remained although still observed throughout the colon they were less dense in the transverse and the ascending colon. By 3 days, they diminished significantly in intensity and in areas of spread and were restricted to the submucosa of the segments in the vicinity of the obstructing ring.

5.3 Ischaemic model

A total of 11 animals were studied. Three animals were sacrificed after the development of ischaemia and the remaining eight animals after resection and anastomosis at different times 3 each after 12h and 24h and 2 at 3 days. Loose adhesions between small and large bowel were more frequently seen in this model than in the other models and the anastomosis appeared intact. Ischaemic segments were cut in to strips and tissue sampled from immediately distal, the next two proximal segments (Prox0 and Prox1), from 25cm from the suture line (Prox2), from the transverse colon (trans) and from the ascending colon (Asc).
5.3.1 Ischaemic segment

The central portion of the ischaemic segment (C) showed the most pronounced changes. In the mucosa, there was loss of the epithelial layer and distortion with haemorrhage of the normal glandular pattern and of the lamina propria. There was loss or displacement of nuclei in surviving glands. The normal connective tissue structure in the submucosal layer was also distorted by haemorrhage and oedema and by focal infiltration of polymorphonuclear leukocytes. In the muscle layer fragmentation of the nuclei and loss of the normal striation pattern were observed. Degeneration of the muscle fibres resulted in the bundles being more spaced out than usual (fig 4.8 b, c, d). With minor variations in extent, this pattern was seen in all animals.

The histological changes in strips A and B were similar to and diminished in severity furthest away from C. Where the damage was less extensive there was only slight oedema, haemorrhage, inflammatory cell infiltration and the epithelial border, mucosal glands and muscle layer were intact (fig 4.9.a, c).

No MMPs were demonstrated in strip C in all 11 animals studied (Table 5.X). In strip A only one rabbit produced extracellular collagenase and stromelysin in the mucosa and intracellular gelatinase in all layers (Table 5.X). However in strip B extracellular MMPs were seen in the mucosa, mainly collagenase but also stromelysin and infrequently gelatinase and TIMP-1 (Table 5.X). Intracellular gelatinase was found in the mucosa of only two animals.
Table 5.X - Ischaemia model
Distribution of MMPs and TIMP-1 in the ischaemic segment after 12 h of ischaemia

<table>
<thead>
<tr>
<th>Tissue segment</th>
<th>Rabbit n°</th>
<th>Mucosa</th>
<th>Submucosa</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Extra</td>
<td>Intra</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>C15, C16, C17, C18, C19, C20, C21, C22, C23, C24, C26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>C15, C16, C17, C18, C19, C20, C21, C22, C23, C24, C26</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Collagenase -  Gelatinase -  Stromelysin -  TIMP -  
Squares - extracellular  Intracellular  Circles -
5.3.2 Tissues from animals after resection and anastomosis

12 h post anastomosis

In the anastomotic segment a large gap between the cut edges of the bowel was filled with a fibrin plug. There was oedema, haemorrhage, polymorphonuclear leukocyte infiltration in the mucosa, the submucosa and the muscle layer near the suture line. In the distal segment the mucosa showed minor abnormalities consisting of slight haemorrhage, oedema and patchy loss of epithelium. Extensive infiltration of polymorphonuclear leukocytes with some oedema were also detected in the submucosa and the muscle layers. The proximal segments were normal except in Prox0 and Proxl where there was oedema and polymorphonuclear cell infiltration of the submucosa with patchy and infrequent epithelial loss.

All the MMPs and TIMP-1 were concentrated in the anastomotic segment and some in the adjacent proximal and distal segments. Extracellular collagenase and stromelysin were intensely fluorescent and widespread in the mucosa and the submucosa of the anastomotic segment. In the mucosa the MMPs were seen in the entire length of this segment but in the submucosa the enzymes were concentrated mainly in the fibrin plug or near the suture line. Extracellular gelatinase and TIMP-1 were also present but not as widely distributed. Intracellular gelatinase was present in all layers of this segment.

Extracellular collagenase and stromelysin were detected in the mucosa of Prox0 and Proxl segments (fig 5.13.a, Table 5.XI). Intracellular gelatinase was detected in all layers of Prox0 and Proxl segments and the distribution of the other enzymes and TIMP-1 was similar but not as consistent. In the Distal segment all the enzymes and TIMP-1 (fig 5.13.b) were present intracellularly in all layers except the muscle layer. The remaining proximal segments did not stain for any of the enzymes (Table 5.XI).
Table 5 XI - Ischaemia model. Distribution of MMPs and TIMP-1 12 h after resection and anastomosis following ischaemia

<table>
<thead>
<tr>
<th>Tissue segment</th>
<th>Rabbit n°</th>
<th>Mucosa</th>
<th>Submucosa</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Extra</td>
<td>Intra</td>
<td>Extra</td>
</tr>
<tr>
<td>Distal</td>
<td>C18, C19, C20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anas</td>
<td>C18, C19, C20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox0</td>
<td>C18, C19, C20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox1</td>
<td>C18, C19, C20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox2</td>
<td>C18, C19, C20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans</td>
<td>C18, C19, C20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asc</td>
<td>C18, C19, C20</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Collagenase - | Gelatinase - | Stromelysin - | TIMP - | Squares - extracellular | Circles - intracellular
24 h post anastomosis

The gap between the cut edges of the bowel wall (fig 5.14.a) was filled with a fibrin clot, haemorrhage, oedema, remnants of mucosal glands, many fibroblasts and mononuclear cells. A large infiltration of polymorphonuclear leukocytes was seen in the fibrin plug extending to the submucosa and the muscle layer. In the distal segment minor disruption of the epithelial border with oedema of the submucosa and infiltration of polymorphonuclear cells of the submucosa, the muscle and the serosa were seen. The changes in Prox0 and Prox1 segments were characterised by oedema and a large polymorphonuclear cell infiltration in the submucosa, the muscle and the serosa. The other proximal segments were normal.

The expression of MMPs and TIMP-1 was still concentrated in the anastomotic segment and the adjacent segments Distal and Prox0 (Table 5.XII). In the anastomotic segment all the enzymes and TIMP-1 were localised extra- and intra-cellularly in the mucosa, the submucosa and in the fibrin plug but not in the muscle layer (fig 5.14.b). As at 12h the expression of MMPs in the mucosa was seen throughout this layer. In the submucosa and in the fibrin plug however, differently from 12h the MMPs and TIMP-1 were not confined to the suture line but spread throughout this segment.

In the distal segment extracellular collagenase, gelatinase and TIMP-1 were present in the mucosa. In the deeper layers all the MMPs and TIMP-1 were present intracellularly and occasionally extracellularly in the submucosa. A similar distribution to the distal segment was also seen in the Prox0 segment. Mainly intracellular enzymes and TIMP-1 were detected in Prox1 and none in the more proximal segments (Table 5.XII).
Table 5.XII - Ischaemia model. Distribution of MMPs and TIMP-1 24 h after resection and anastomosis following ischaemia

<table>
<thead>
<tr>
<th>Tissue segment</th>
<th>Rabbit n°</th>
<th>Mucosa</th>
<th>Submucosa/fibrin plug</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Extra</td>
<td>Extra</td>
<td>Extra</td>
</tr>
<tr>
<td>Distal</td>
<td>C21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anas</td>
<td>C21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox0</td>
<td>C21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox1</td>
<td>C21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox2</td>
<td>C21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans</td>
<td>C21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asc</td>
<td>C21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Collagenase - □ Gelatinase - □ Stromelysin - □ TIMP - □ Squares - extracellular Circles - intracellular
3 days post anastomosis

In the anastomotic segment the fibrin plug had increased in size and was filled with inflammatory cells and fibroblasts (fig 5.15.a). Around the fibrous tissue and in the submucosa there was much less oedema and inflammation. There were minor changes only in distal and Prox0 segments which consisted of slight oedema, haemorrhage and polymorphonuclear leukocyte infiltration. The other segments were normal.

In the mucosa of the anastomotic segment intra- but not extra-cellular collagenase, stromelysin and gelatinase were present whereas in the fibrous plug all MMPs and TIMP-1 were present both extra- and intra-cellularly. Occasional cells containing intracellular enzymes were seen in the different layers of the Distal and Prox0, but none in the other segments (Table 5.XIII).

5.3.3 Summary of the results in the ischaemic model

In the resected ischaemic segment the maximum changes was in the central portion where there was loss of the epithelial border and destruction of the normal glandular structure and the deeper layers with fragmentation and degeneration of the muscle fibres. The changes were less marked at the margins of this segment.

No MMPs were detected in the central portion and in the margins intra- and extra-cellular expression was inconsistent and was seen in few specimens, probably related to variation in the degree of ischaemia.

In the anastomotic segments, the histological changes 12h and 24h post-anastomosis were similar, between the cut ends the bowel was filled with fibrin clot and there was haemorrhage, oedema and polymorphonuclear leukocyte infiltration of all the layers especially near the suture line. The distal and adjacent proximal segments showed
<table>
<thead>
<tr>
<th>Tissue segment</th>
<th>Rabbit n°</th>
<th>Mucosa</th>
<th>Submucosa/fibrous tissue</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Extra</td>
<td>Extra</td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td>C24</td>
<td>⬜️</td>
<td>⬜️</td>
<td>⬜️</td>
</tr>
<tr>
<td></td>
<td>C26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anas</td>
<td>C24</td>
<td>⬜️</td>
<td>⬜️</td>
<td>⬜️</td>
</tr>
<tr>
<td></td>
<td>C26</td>
<td>⬜️</td>
<td>⬜️</td>
<td>⬜️</td>
</tr>
<tr>
<td>Prox0</td>
<td>C24</td>
<td>⬜️</td>
<td>⬜️</td>
<td>⬜️</td>
</tr>
<tr>
<td></td>
<td>C26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox1</td>
<td>C24</td>
<td>⬜️</td>
<td>⬜️</td>
<td>⬜️</td>
</tr>
<tr>
<td></td>
<td>C26</td>
<td>⬜️</td>
<td>⬜️</td>
<td>⬜️</td>
</tr>
<tr>
<td>Prox2</td>
<td>C24</td>
<td>⬜️</td>
<td>⬜️</td>
<td>⬜️</td>
</tr>
<tr>
<td></td>
<td>C26</td>
<td>⬜️</td>
<td>⬜️</td>
<td>⬜️</td>
</tr>
<tr>
<td>Trans</td>
<td>C24</td>
<td>⬜️</td>
<td>⬜️</td>
<td>⬜️</td>
</tr>
<tr>
<td></td>
<td>C26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asc</td>
<td>C24</td>
<td>⬜️</td>
<td>⬜️</td>
<td>⬜️</td>
</tr>
<tr>
<td></td>
<td>C26</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Collagenase - □ Gelatinase - □ Stromelysin - □ TIMP - □ Squares - extracellular Circles - intracellular
some oedema and inflammatory cell infiltrate but the other segments were normal. By 3 days a fibrous plug had formed and there was much less oedema and inflammatory changes in the anastomotic segment and its adjacent segments.

At 12h extracellular and intracellular collagenase and stromelysin as well as TIMP-1 were widespread in the mucosa and the submucosa of the anastomotic segment, concentrated round the suture line. At 24h the MMPs and TIMP-1 were not confined to the suture line but were diffuse throughout the anastomotic segment. In the deeper layers intracellular gelatinase was a constant feature. By 3 days, the extracellular and intracellular MMPs and TIMP-1 were mainly in the fibrous plug only intracellular MMPs were present in the mucosa. In the distal and the immediate proximal segments intracellular MMPs, mainly gelatinase were present. The more proximal segments did not stain for MMPs.
Figure 5.1 - Normal colon

Figure shows the rabbit normal distal colon at the site of anastomosis
m-mucosa  mm-muscularis mucosae  sm-submucosa  mu-muscle
s-serosa  Original magnification x 40
12h after anastomosis a fibrin plug is forming between the cut edges of the bowel. In this area, inflammatory cell infiltration can be seen (see fig 4.2.c). Slight haemorrhage in all layers is also observed. Oedema of the submucosa extends a few mm either side of the suture line. Original magnification x 25

m-mucosa sm-submucosa mu-muscle fp-fibrin plug.

Diagram showing the distribution of
Collagenase TIMP Gelatinase Stromelysin.

12 h after transection and anastomosis the MMPs and TIMP all localised to the cut edges of the bowel wall predominantly in the mucosa.
Figure 5.2. Normal healing model. 12 h after operation 
Figures showing anastomotic segment

c) Original magnification x 100

d) Original magnification x 200

The two figures show details of the fibrin plug where inflammatory cell infiltration is seen. At this time polymorphonuclear cells predominate.
m-mucosa  fp-fibrin plug  mu-muscle
Figure 5.2 - Normal healing model. 12 h after operation. Figures e to h are all the same anastomosis segment stained by indirect immunofluorescence for the MMPs. The nuclei have been counterstained to fluoresce red.

e) Original magnification x 400

Inverted mucosa in the anastomotic segment, uncultured tissue incubated with the antiserum to collagenase showing the enzyme associated with the extracellular matrix (arrows) surrounding the mucosal glands.

f) Original magnification x 400

The muscle layer stained with antiserum to collagenase, which is seen as streaks of green fluorescence (arrows) near the muscle bundles.
Tissue in g) was incubated with antiserum to gelatinase and tissue in h) with antiserum to stromelysin. Gelatinase and stromelysin can be seen as streaks of green fluorescence (arrows) associated with the extracellular matrix surrounding the mucosal glands.
At 24 h a fibrin plug fills the gap between the bowels end. More inflammatory cells have infiltrated than at 12 h, mainly in the submucosa and around stitches. The oedema and haemorrhage is more extensive than at 12 h.

m-mucosa sm-submucosa mu-muscle fp- fibrin plug. Original magnification x 20

Diagramatic representation of the distribution of Collagenase TIMP Gelatinase and Stromelysin 24 h after transection and anastomosis. MMPs and TIMP predominantly localised to the cut edge although in the mucosal layer they are seen to extend a few mm from the stitch line.
After 3 days fibrous tissue infiltrated by a large number of acute and chronic inflammatory cells fills the gap between the cut edges of the bowel. Muscle (little arrow) and mucosal remnants (open arrow) are seen in this region. A connective tissue layer seals the serosal surface (arrow).  

m-mucosa  sm-submucosa  mu-muscle  ft-fibrous tissue.  
Original magnification x 25

b)

The distribution of Collagenase TIMP Gelatinase Stromelysin

3 days after transection and anastomosis is shown diagramatically. MMPs and TIMP are still both found at the cut edge with more enzyme now seen in the muscle layer.
Figure 5.5 - Normal healing model. Anastomosis 7 days after operation
Figures of anastomotic segment

a) Formalin fixed tissue stained with H&E

Mucosal continuity is almost restored. A mature fibrous tissue still contains some buried mucosal crypts and muscle cells (see fig. 4.5.c). The serosal surface is sealed by a connective tissue layer (arrow).
m-mucosa  sm-submucosa  mu-muscle  ft-fibrous tissue  s-stitches
Original magnification x 25

b)

Distribution of
Collagenase  TIMP  Gelatinase  Stromelysin
represented diagrammatically with the dots indicating intracellular MMPs and TIMP. Most enzyme is seen in the deeper layers of the bowel wall.
Figure 5.5 - Normal healing model
Figures show details of the anastomotic segment 7 days after anastomosis formation. Formalin fixed tissues stained with H&E

**c)** Original magnification x 100

Large number of inflammatory cells infiltrating the fibrous tissue where muscle and mucosal remnants (arrows) remain to be degraded.

**d)** Original magnification x 200

The inflammatory infiltrate around muscle remnants is a mixed cell population. In this photograph both multinucleated and mononuclear cells are visible.
Figure 5.5 - Normal healing model. Anastomosis 7 days after operation. Figures of anastomotic segment stained with gelatinase or stromelysin using indirect immunofluorescence with the nuclei counterstained to fluoresce red.

e) Original magnification x 400

Figures show the fibrous tissue near the tip of the inverted mucosa. Tissue in e) was incubated with antiserum to gelatinase and shows intracellular granules (arrows). Intracellular stromelysin was also seen in the same region when the tissue was incubated with antiserum to stromelysin f) (arrows).
Figure 5.5 - Normal healing model. Anastomosis 7 days after operation
Figure of anastomotic segment. Stromelysin immunolocalisation.

**g)** Original magnification x 400

Figure shows the tip of the cut edge of the inverted mucosa stained with antiserum to stromelysin. Streaks of green immunofluorescence (arrows) demonstrate the presence of extracellular stromelysin around mucosal glands. The nuclei have been counterstained to fluoresce red.
At 12 h the gap between the cut ends of the bowel is filled with clot, oedema and detached fragments of muscle and mucosa. A large infiltration of inflammatory cells can be seen in the anastomotic region (see detail in fig 5.6.c and d)

m-mucosa  sm-submucosa  mu-muscle  Original magnification x 25

Diagram of Collagenase TIMP Gelatinase and Stromelysin distribution. Intracellular gelatinase is represented by dots (○) seen in many cells in the submucosa. Extracellularly, collagenase was the most widespread MMP in the mucosa and submucosa.
Figure 5.6 - Obstruction model

Figures show detail of the anastomotic segment 12 h after resection of the segment containing the ring and anastomosis formation. Formalin fixed tissues stained with H&E.

c) Original magnification x 100
d) Original magnification x 200

Note the large number of inflammatory cells infiltrating all layers of the bowel wall in c) and d). Both mononuclear and polymorph cells are present. In c) almost complete loss of the normal gland pattern has occurred and the epithelial cell border is absent.

m-mucosa  sm-submucosa  mu-muscle  s-serosa
The figures show the mucosa in the anastomotic segment. In e) the tissue was incubated with antiserum to collagenase and in f) with antiserum to TIMP-1. Extracellular collagenase and TIMP-1 is demonstrated as streaks of green fluorescence around mucosal glands (arrows). Note that collagenase is much more bright and widespread than TIMP-1. The nuclei have been counterstained to fluoresce red.
Both figures show the mucosa of the anastomotic segment. In g) the tissue was incubated with antiserum to gelatinase and in h) with antiserum to stromelysin. Both enzymes were immunolocalised extracellularly, demonstrated by bands of green fluorescence around the mucosal glands (arrows). The nuclei have been counterstained to fluoresce red.
Figure 5.6. Obstruction model. Figures show tissue taken 12 h after operation.

i) Original magnification x 1000.

Figure shows the submucosa of the anastomotic segment incubated with antiserum that recognises gelatinase B. Brightly intracellular fluorescent granules were immunolocalised in cells consistent with polymorphonuclear leukocytes (arrows). The nuclei have been counterstained to fluoresce red.

j) Original magnification x 1000

Figure shows the submucosa of the distal segment from the same rabbit, incubated with antiserum to gelatinase A and B. Green fluorescent staining marks the presence of the enzyme associated with a solitary macrophage-like cell. Red fluoresce are the nuclei.
Figure 5.6. Obstruction model. Figures show tissue taken 12 h after operation.

**k)** Original magnification x 400

Lower power of the two preceding figures demonstrating the large number of cells expressing intracellular gelatinase (arrows). The tissue was incubated with antiserum that recognises gelatinase A and B.
Figure 5.7. Obstruction model
Figures show the Prox2 segment 12 h after resection of the ring and anastomosis formation

a) Original magnification x 100

b) Original magnification x 200

Typical alterations observed at 20 cm proximal to an anastomosis formed 12 h before, following resection of the segment containing the obstructing ring. Massive oedema of submucosa and muscle and inflammatory cell infiltration in all layers are seen. The mucosa shows the heaviest infiltration and also superficial ulceration represented by destruction of the epithelial border. m-mucosa sm-submucosa mu-muscle
Figure 5.8 - Obstruction model.

Figures show the anastomotic segment 24 h after resection of the obstruction and anastomosis formation.

a) Formalin fixed tissue stained with H&E

At 24 h there was still a large gap between the cut edges of the bowel filled with clot, fibrin, oedema and detached inverted muscle. Note the oedematous submucosa and the heavy infiltration of inflammatory cells mainly in the fibrin plug, in the submucosa and around stitches. Original magnification x 25.

m-mucosa  sm-submucosa  mu-muscle  fp-fibrin plug  s-stitches.

b)

Collagenase  TIMP  Gelatinase  and Stromelysin

are represented diagrammatically. Dots (○) show intracellular gelatinase, mainly in the submucosa whereas extracellular enzyme is seen in the mucosal layer.
Stitches are seen to be surrounded by large numbers of inflammatory cells 24 h after anastomosis following an obstruction.

mu-muscle  se-serosa  st-stitches
After 3 days, similarly to the normal healing model, a large oedematous fibrous tissue, heavily infiltrated by inflammatory cells occupied the gap between the bowels ends. At this site mucosal and muscle remnants undergoing degradation are also present. Original magnification x 25

m-mucosa  sm-submucosa  mu-muscle  ft-fibrous tissue.

Collagenase TIMP  Gelatinase and Stromelysin
distribution shown diagrammatically. ○○ represent intracellular gelatinase which is the dominant feature. Extracellular enzyme is restricted to the stitch line.
Figure 5.9 - Obstruction model.
Figures 5.9 c to f are all from the anastomotic segment of the same rabbit 3 days after resection of the obstructed segment and anastomosis. The sections have been stained by indirect immunofluorescence for the MMPs and TIMP-1 including a counterstain for the nuclei which fluoresces red.

c) Original magnification x 400

![Image](image1)

In c) the tissue was incubated with antiserum to collagenase and in d) with anti TIMP-1. In the mucosa both the enzyme and the inhibitor were observed as strips of green fluorescence (arrows) surrounding mucosal glands.

d) Original magnification x 400

![Image](image2)
Figure 5.9. Obstruction model. Figures show the anastomotic segment 3 days after operation.

\(e\) Original magnification x 400

The section in \(e\) was stained with antiserum to gelatinase and the section in \(f\) was incubated with antiserum to stromelysin. Both enzymes were immunolocalised as streaks of green fluorescence around the mucosal glands (arrows). Note that the TIMP-1 staining (d) is much less extensive and duller than that of the MMPs.
Figure 5.10 - Obstruction model.
Figures are the anastomotic segment 7 days after resection of the obstruction and formation of anastomosis.

a). Formalin fixed tissue stained with H&E

7 days after anastomosis, the mucosal continuity is almost restored and a mature fibrous tissue has formed. Oedema and inflammatory cell infiltration are still seen in the anastomotic region. Original magnification x 25.

m-mucosa sm-submucosa mu-muscle ft-fibrous tissue s-stitches

b).

Collagenase TIMP Gelatinase and Stromelysin
distribution at the anastomosis. By 7 days MMPs and TIMP are only present at the stitch line.
Figure 5.11 - Obstruction model.

Figures show ProxO segment after 24 h of obstruction

a) Formalin fixed tissue stained with H&E

In the mucosa on the left side of the photo extensive crypt damage and loss of epithelial border have occurred (fig 5.11.c,d). Oedema, inflammatory cell infiltration and haemorrhage can also be seen throughout the segment especially in the submucosa and muscle.

m-mucosa  sm-submucosa  mu-muscle. Original magnification x 40

b)

Diagram showing distribution of
Collagenase  TIMP  Gelatinase  and Stromelysin

in the ProxO segment. Where the epithelial border is damaged extracellular enzyme is seen whereas MMPs and TIMP are seen in the other layers but only intracellularly (○○)
Figure 5.11. Obstruction model
Figures show the Prox0 segment after 24 h of obstruction at the site of most extensive mucosal damage.

c) Original magnification x 100

Both figures are details of fig 5.11.a. showing the extensive superficial ulceration evidenced by loss of the epithelial border and many crypts. Note the heavy infiltration of inflammatory cells at these sites. This was characteristic of the mucosal damage seen in the most proximal segments of the obstruction model.
m-mucosa  mm-muscularis mucosae  sm-submucosa
A section showing the submucosal layer incubated with antiserum to gelatinase A and B. The presence of intracellular gelatinase is demonstrated by green fluorescent granules near the nuclei (counterstained to fluoresce red) of a large number of cells. The elongated nuclei showing granules at one extremity appear to be fibroblast-like cells (thin arrows). The cells with granules surrounding the nucleus may be polymorphonuclear leukocytes (large arrows).
Figure 5.12 - Obstruction model. Prox1 segment after 24 h of obstruction

a) Formalin fixed tissue stained with H&E

Note the extensive damage of the mucosa manifested by total loss of the epithelial border and destruction of crypts. Inflammatory cell infiltration (fig 5.12.c,d), oedema and haemorrhage in all layers can also be seen. m-mucosa sm-submucosa mu-muscle Original magnification x 40

b) Collagenase TIMP Gelatinase and Stromelysin distribution in Prox1 segment. Extracellular MMPs dominate in the mucosa whereas only intracellular gelatinase (○) is observed in the other layers.
Figure 5.12. Obstruction model
Figures are magnification of areas from fig. 5.12.a.

c) Original magnification x 100

Note the extensive infiltration of inflammatory cells in all layers of the bowel wall especially in the mucosa where many epithelial glands are missing.
m-mucosa  mm-muscularis mucosae  sm-submucosa  mu-muscle

d) Original magnification x 200

Detail of the preceding figure showing loss of the epithelial border of the mucosa, gland destruction and heavy inflammatory cell infiltration, containing both acute and chronic cell types.
m-mucosa  mm-muscularis mucosae  sm-submucosa  mu-muscle
Figure 5.13 - Ischaemic model. Figures show tissue taken 12 h after anastomosis

**a)** Original magnification x 400

Figure shows the mucosa of the Prox0 segment incubated with antiserum to stromelysin. Bands of green fluorescence demonstrate the binding of stromelysin to the ECM (arrows). The nuclei have been counterstained to fluoresce red.

**b)** Original magnification x 400

Tissue taken from the distal segment incubated with antiserum to TIMP-1. The inhibitor is seen intracellularly surrounding the nuclei which fluoresce red in the submucosa (arrows).
24 h after anastomosis the large gap between the cut edges of the bowel was filled with fibrin, haemorrhage, oedema, remnants of mucosal glands and muscle. A large number of inflammatory cells have infiltrated the fibrin plug extending to the submucosa and muscle. Original magnification x 25
m-mucosa sm-submucosa mu-muscle fp-fibrin plug s-stitches

Diagrammatic representation of Collagenase TIMP Gelatinase and Stromelysin at the anastomosis. Intracellular gelatinase (○ ○) dominates accompanied by extracellular MMPs restricted to the mucosa.
Figure 5.15 - Ischaemic model.

Anastomotic segment 3 days after resection and anastomosis.

a) Formalin fixed tissue stained with H&E

3 days after anastomosis a fibrous tissue fills the gap between the cut bowel ends. It is infiltrated by many inflammatory cells and also contains remnants of mucosal glands and muscle. Original magnification x 25

m - mucosa  sm - submucosa  mu - muscle  ft - fibrous tissue

b)

Collagenase  TIMP  Gelatinase  and  Stromelysin
distribution shown diagrammatically at the anastomosis. Both intra- and extra-cellular MMPs and TIMP occur only at the suture line.
CHAPTER 6

DISCUSSION

6.1 Introduction

Halsted in 1887 was the first to realise that the submucosal layer was the layer which contributed most to anastomotic strength. Collagen is the major component of the submucosa and Cronin et al (1968) showed that the reduction in bursting strength of the colon during the first few days following an anastomosis correlated with the decrease in the concentration of collagen. Dunphy (1970), Irvin and Hunt (1974) and Jiborn et al (1978, 1980a) further confirmed these observations. Hawley 1970 suggested that the reduction in collagen was due to collagenase activity but this was measured by an indirect method. However Chowcat et al (1988) could not demonstrate, by immunohistochemistry, excessive collagenase in a normal colonic healing model. Since these studies other matrix metalloproteinases, namely stromelysin and gelatinase, have been identified which together degrade not only collagen but all the components of the extracellular matrix (Murphy and Reynolds, 1993).

Several risk factors have been implicated in anastomotic dehiscence (Morgenstern et al 1972, Schrock 1973) but even when these are avoided and the surgical technique is perfected, anastomotic dehiscence still occurs. Hence clinicians have attempted to improve colonic healing biochemically by the use of aprotinin, a proteolytic enzyme inhibitor (Young and Wheeler 1984, Sheridan et al 1989). The results, however, were disappointing but have directed attention to the need to clarify the biochemical events in colonic healing.
6.1.1 Methodology

An indirect immunofluorescent technique was chosen to study the matrix metalloproteinases because they are produced in small quantities only on demand and are likely to be strictly localised in selected areas. Biochemical analysis requires a relatively large amount of tissue. Therefore any localised change may not be recognised as deviation from normality within a large sample as detection of small changes can be difficult. Furthermore for biochemical analysis the tissue is homogenised and this would not allow the localisation of the enzymes and TIMP to be determined.

The immunofluorescent technique used in this study has the advantage of being extremely sensitive as is shown by its ability to identify granules within cells (intracellular enzyme or TIMP) as well as the enzyme and inhibitor on the matrix (extracellular enzyme or TIMP). Localisation of MMPs and TIMP is crucial as their production is related to the microenvironment. For example, in unimpaired colonic healing the matrix metalloproteinases extend only a millimetre away from the cut edges of the bowel. As was shown by Chowcat et al (1990) in order to measure collagenase levels in normal colonic healing, culture of the tissue is required which stimulates the enzyme activity even in normal colon where immunolocalisation has shown that the enzymes are not present. It has been pointed out that the levels measured in culture media may only reflect the overspill after tissue degradation, thus not representing the true tissue enzyme levels (Fell et al, 1989). The measurements by Chowcat et al (1990) of collagenase appeared to reflect the effect of culture on the colon rather than the result of the anastomosis. Biochemical assays have to be supplemented by SDS-PAGE electrophoresis to demonstrate the specificity of the activity being measured, an essential step which was omitted by van der Stappen et al (1989).
The disadvantage of immunohistochemistry is that it does not provide a quantitative measurement of collagenase or other MMPs although with the small quantities produced such measurements can be difficult. The antisera used in this study detect the pro-MMP, the active form and the enzyme-TIMP complexes. Therefore the MMPs when immunolocalised can be in any of these forms. However only active collagenase can bind to the matrix, whereas TIMP-1 does not bind to the matrix unless attached to active collagenase, gelatinase A or stromelysin (Allan et al, 1991, J.A. Allan and G. Murphy, personal communication).

To investigate the matrix metalloproteinases in models of anastomotic healing, the distribution of the enzymes and their inhibitor is best determined by immunohistochemical localisation because of its sensitivity, specificity and the importance in these models of determining the distribution of the enzymes in relation to the stitch line. Although not carried out in this study, double-labelling for cell markers can be employed to identify the cell type synthesising the matrix metalloproteinases.

6.2 Normal healing model

In the normal healing model histological abnormalities were confined to the suture line. This too was the only region in which MMPs and TIMP-1 were seen. Neither the enzymes or TIMP-1 were ever present away from the stitch line or in normal tissue. This was confirmed in rabbits having a sham operation and the handling of the colon did not influence the expression of these enzymes or their inhibitor. It is not surprising that the MMPs are absent in normal colonic tissue as the turnover of the extracellular matrix components is slow. Furthermore, these enzymes are not constitutively expressed but are only synthesised on demand with the exception of the collagenase and the gelatinase stored in macrophages and polymorphonuclear leukocytes.
The distribution of the MMPs is a direct reflection of the histological abnormalities seen on haematoxylin and eosin staining. The mucosal layer, with its predominance of MMPs in the first 24 hours is the layer which disrupts and heals most rapidly. The enzymes are seen only at the cut edges which is the area of damaged mucosa requiring remodelling for a continuous mucosal layer to be reformed. The damaged mucosa was seen to be removed by the seventh day and in some cases epithelial cells had migrated across the gap to form a complete epithelial border. In residual areas of mucosal abnormalities MMPs were still present.

Initially the gap created by the transection was filled by a clot in the submucosal and muscle layers, together with some fragments of detached mucosa and muscle and was seen to be infiltrated by polymorphonuclear cells. At this stage very little MMP and TIMP activity was present in this area. As the gap filled with new connective tissue this area became the focus for MMP activity. Eventually this tissue contracts to a narrow scar line (Stein and Barry 1983, Foster and Leaper 1984, Hesp et al 1985). Indeed, by the seventh day the fibrous tissue became more collagenous and compared to the third day much less extracellular MMPs and TIMP were seen; most of the enzymes and inhibitor were confined within cells. This pattern observed on the seventh day probably indicates a very gentle remodelling process which continues until either a normal structure is reformed or a mature scar has formed. In corneal wounds stromelysin and collagenase have been seen for up to seven months (Girard et al, 1993).

There has been only one previous immunohistochemical study in colonic anastomosis by Chowcat et al (1988). Using a rabbit model of everted colonic anastomosis Chowcat et al (1988) studied the role of collagenase and TIMP-1 in the healing process from 12h to 7 days after anastomosis. They found collagenase activity confined to a narrow margin at the cut ends of the bowel wall and showed that after 7 days both enzyme and inhibitor were active only in small areas where dying tissues remained to be destroyed and where remodelling was taking place. There was no
evidence for excessive collagenase activity in normal colonic anastomotic healing. Indeed the inhibitor was always found at the same site as collagenase. These observations agree with this study using the same method and antibodies but with an anastomosis formed with extra-mucosal sutures. Furthermore for the first time gelatinase and stromelysin have been immunolocalised in colonic anastomosis.

The only other study which specifically measured collagenase was reported by Van der Stappen et al (1992). They demonstrated that extracts of ileal and colonic anastomosis degraded type I and III collagen producing the characteristic 3/4, 1/4 fragments on SDS-PAGE electrophoresis, which are evidence of mammalian collagenase activity. Their studies also showed that collagenase activity was much higher round a colonic anastomosis compared to an ileal anastomosis. The activity was measured in a homogenised tissue with the stitch line in the centre of a 1cm segment but not in any other segments. This increased collagenase activity at the anastomosis mirrored the immunohistochemical localisation of the MMPs to the suture line seen in this study.

The lack of MMPs in normal tissue conflicts with the results of Hawley (1970) who reported higher levels of collagenolytic activity in rabbit colon than other regions of the gastrointestinal tract. These levels rose even further following sham laparotomy which also contradicts this study. However it is infeasible to assume from Hawley’s work that the enzyme activity he measured was specifically due to collagenase. His observations were based on measurements of the area of lysis of collagen gels produced by colonic explants but he did not prove that this activity was mammalian collagenase. The lysis of collagen gels could have been caused by bacterial collagenase or other proteases. Cathepsin G, cysteine proteases and elastase can degrade extracellular matrix when the appropriate conditions are present; for example an alkali pH will allow elastase activity and an acidic pH cysteine protease activity. While their usual role is the intracellular digestion of phagocytosed fragments under unphysiological pH they may act extracellularly (Murphy and Reynolds, 1993). It is conceivable that any mammalian
collagenase activity measured was induced by the culture conditions as Chowcat et al (1990) have shown that colonic biopsies of normal rabbit colon maintained in culture secreted collagenase into the media following a lag period of 24 hours when the enzyme was undetectable by immunohistochemistry.

The restricted expression of the matrix metalloproteinases to the immediate vicinity of the anastomoses and the presence of TIMP-1 at the same location presented in this thesis also conflicts with the reports (Cronin et al 1968, Jibom et al 1980a, b Hesp et al 1984, Hendricks et al 1985) that collagen concentration is decreased in the anastomotic segment extending for several centimetres proximally. The discrepancy between the limited localisations of MMPs and the more extensive reduction of collagen may be due to other proteolytic enzymes, e.g. cathepsins, as previously explained (Murphy and Reynolds 1985a). Van der Stappen (1989) reported increased collagenolytic activity in extracts of tissue from anastomoses and from tissue taken proximally. The more widespread activity measured in the first 24h following an anastomosis suggests that other enzymes could be involved at least in the initial period, although the specific enzymes could not be determined from Van der Stappens study.

Other investigations indicate, however, that measurement of collagen concentration is not representative of the total amount of collagen, but could be related to accumulation of non-collagenous proteins resulting in an apparent reduction in collagen concentration while the true amount is unchanged (Tornqvist et al, 1988, Jonsson et al 1986, Stromberg and Klein 1982). Stromberg and Klein (1982) who measured collagen concentration, following formation of a colonic anastomosis, per unit length demonstrated that colonic collagen mass is not decreased. They found new collagen, demonstrated by uptake of radioactivity, only at the suture line and within a few millimetres either side. Similarly in this study the histological changes and the immunohistochemical distribution of MMPs and TIMP-1 were restricted to the stitch line. An immunohistochemical study of collagen types in the colonic submucosa (Brasken
1989) showed diminished staining for collagen I and III in the first two days and an increase from the third day following an anastomosis. Hesp et al (1984) pointed out that the decrease in collagen demonstrated by fluorescence could be due to oedema of the tissue rather than an actual reduction in collagen. Tornqvist et al (1988) also reported that collagen concentration can be a misleading indicator of the actual amount of collagen as they found an increase in collagen content in rats with colonic stenosis when the collagen concentration was decreased. Therefore the deductions from these studies is that the reduction in collagen proximal to the anastomoses could be an artefact of measurement rather than a real reduction in its level.

The findings in the normal healing model of collagenase, stromelysin and gelatinase localised to the suture line with their inhibitor, TIMP-1 present in the same area are taken as the standard healing pattern. Their presence, limited to the immediate region of the anastomosis, where the tissue has been disrupted and degradation is needed before the normal bowel structure can be restored, suggests that the MMPs are involved in colonic healing in a well controlled process.

6.3 Obstruction model

6.3.1 Obstruction only

Following obstruction extensive histological as well as immunohistochemical changes to all layers of the bowel wall and extending beyond the site of obstruction both distally and proximally were evident.

An obstruction resulted in damage to the mucosa which manifested as loss of the epithelial border, some destruction of the crypt architecture, haemorrhage, oedema and infiltration of acute inflammatory cells. These changes occurred not only at the site of the obstruction but were seen to extend to the distal and the adjacent proximal segments,
although in the segment taken 25cm from the obstruction, equivalent to the level of the descending colon there were only minimal abnormalities. There was however a very rapid return to normality as by 24h after the obstruction was released the histological abnormalities seen earlier had abated considerably and by the third day had resolved. This pattern of changes was reflected in the expression of the metalloproteinases which were seen extracellularly on the mucosal matrix only in those rabbit killed immediately after 24h of obstruction that is when damage was maximal. TIMP-1 was frequently absent suggesting that degradation was progressing. However, the rapid return to a normal histology implies that together with the high mucosal cell turnover, synthesis of extracellular matrix components must be occurring at an even faster rate.

The enzymes were present in obstructed segments of bowel but surprisingly despite similar histological changes at the site of obstruction (ring site) the expression of MMPs was different. There was only intracellular gelatinase probably associated with infiltrating polymorphonuclear cells which were abundant at this site as well as resident by fibroblast-like cells. Lack of histological change when a large ring was left in contact with the colon make it unlikely that was toxic inhibition of MMP expression. By 24h after release of the obstruction only intracellular enzymes were present predominantly gelatinase which is thought to be the “housekeeping enzyme” (G. Murphy, personal communication). The intracellular enzyme seen is probably accompanied by an extremely low level of extracellular secretion which is not visible by light microscopy but sufficient for the level of degradation still required for complete restoration of the mucosa. When the mucosa was normal on the third day no MMPs or TIMP were seen.

In the submucosa, the muscle and the serosa the damage was characterised by oedema, haemorrhage and infiltration of numerous polymorphonuclear leukocytes which was maximal immediately after 24h of obstruction and diminished once the obstruction was released. By the third day the only residual abnormality was infiltration of a few inflammatory cells. These changes, as in the mucosa, extended away from the site of
obstruction segment with occasional oedema of the submucosa as far as the transverse colon. At no time in these layers were the MMPs or TIMP-1 seen extracellularly but were present intracellularly throughout the study period. Although all the MMPs were seen intracellularly the most predominant enzyme was gelatinase especially by the third day. The intracellular expression occurred not only in the obstructed segment but also extended to the ascending colon where the colon appeared normal. However, by the third day following the release of the obstruction the intracellular enzymes were restricted to those segments immediately proximal to the obstruction and only very few cells were seen synthesising the enzymes or TIMP. By this time the enzymes were restricted to the submucosa while at earlier periods they had also been seen in the muscle and serosa.

The presence of intracellular enzymes in the ascending colon for the first 24h following an obstruction suggests that some minor damage had occurred in this region although histologically it appeared normal on light microscopy. It is possible that colonic dilatation, diminished blood perfusion and faecal loading induced by obstruction stimulate MMP synthesis. At present, although in vitro, many factors have been shown to stimulate production of the MMPs and TIMP-1 very little is known of the mechanism of stimulation in vivo. The dominance of gelatinase is probably related to its "housekeeping role" and to its stimulatory pathway which is different from that of the collagenases and stromelysins (6.6.3).

The single study of colonic obstruction in an experimental model was reported by Tornqvist et al (1988). A stenosis in the distal colon of rats was found to stimulate the synthesis of collagen at the stenotic site and immediately proximal to it when compared to normal colon. The collagen content was increased proximal to the stenosis however at the stenotic site there was no difference from the levels in normal colon. As synthesis was increased, the finding of no change in collagen content at the stenotic site implies that there must also be an equivalent increase in degradation which is consistent with the increase in metalloproteinases reported in this thesis. The increased collagen content
proximal to the stenosis appears to contradict this study because, there was still a demonstrable increase in MMPs in the same area. However a raised synthesis and content does not preclude degradation occurring simultaneously, but only indicates that overall the rate of synthesis is greater than that of degradation. Indeed the very rapid return to a normal histology and the transient expression of extracellular MMP does suggest that synthesis levels are high in this model. Stromberg and Klein (1982) also showed by isotope labelling an increase in the total collagen following obstruction of the small intestine which was due to deposition of new collagen mainly at the site of obstruction without breakdown of the old collagen. The apparent lack of collagen degradation may be due to differences in collagen metabolism between the small and large bowel.

This study and the one of Tornqvist and his colleagues (1988) both demonstrate that in the obstructed bowel, collagen metabolism is disturbed and this is not localised to the obstructed site but is widespread. Whether the factors that stimulate these changes are related to mechanical effects due to the distension of the bowel remain to be elucidated. Obstruction affects intestinal blood flow with a decrease in flow reported in models of complete acute obstruction (Shikata et al 1983, Ruf et al, 1980, Boley et al 1969) whereas in partial, chronic obstruction the blood flow is increased in the proximal obstructed colon (Papanicolaou et al 1989, 1985, Coxon 1984). It is a matter of speculation in this model as the blood flow was not measured. Changes in blood flow may have contributed to the pattern of expression of MMPs observed in the obstructed colon.

6.3.2 Obstruction and anastomosis

Unlike the normal healing model, the histological abnormalities were not restricted to the anastomotic site but extended proximally to involve the entire descending colon. The changes persisted to the third post-operative day, but by the seventh day were
confined to the anastomotic segment as in the normal healing model. The widespread
damage to the descending colon was associated with the expression of the matrix
metalloproteinases which persisted beyond the anastomotic segment for three days after
an anastomosis was carried out. Otherwise the changes at all times at the suture line both
histologically and in the expression of the MMPs and TIMP-1 were identical to the
normal healing model already described and discussed (6.2).

The mucosa at 12h post-anastomosis exhibited patchy loss of the epithelial border
with infiltration of inflammatory cells, haemorrhage and oedema. These changes were
seen in the entire descending colon but were most extensive in the anastomotic segment,
involving the entire section not just at the suture line, but proximally and to a lesser extent
distally. This marked damage to the mucosa was accompanied by extracellular
expression of all the MMPs in the mucosa, only in those areas in which the epithelial
border had been lost. While TIMP-1 was scarce in this segment of the colon in the rabbits
killed immediately after anastomosis, TIMP-1 usually co-localised with the MMPs,
when the anastomosis was examined after 12h. At 24h post-anastomosis the only
histological difference was an increase in the number of inflammatory cells infiltrating the
mucosa and there was little change in the pattern of MMP and TIMP-1 expression in this
layer. By the third day the histological changes although still present were considerably
milder than at earlier times with reduction of MMP expression especially collagenase in
the mucosa although interestingly, and for no apparent reason, this did persist in the
furthest segment (Prox 2). TIMP-1 was always present in the same area indicating that
any active enzyme was likely to be rapidly inhibited. The return to a completely normal
histology on the seventh post-operative day was shown by the MMPs and TIMP-1 only
occurring at the stitch line as in normal healing.

The major histological changes in the other layers was oedema and inflammatory
cell infiltrate, which increased from 12h to 24h post-anastomosis but decreased to fewer
cells and much less oedema on the third post-operative day. Away from the stitch line
only intracellular enzymes were observed which in rabbits killed from 12h to 3 days post-anastomosis were exclusively gelatinase. It is conceivable that oedema distorts the collagen fibres in the submucosa and their remodelling is provided by gelatinase. The presence of predominantly polymorphonuclear cells which are rich in gelatinase suggests that these cells may be involved in the remodelling of the submucosa.

No previous studies have examined the matrix metalloproteinases in an anastomosis following an obstruction, but collagen measurements have demonstrated collagen lysis at the anastomosis. Jiborn et al (1978) found that continuous sutured anastomosis in rats resulted in a partial obstruction and a marked decrease in collagen concentration compared to interrupted sutured anastomosis. Tomqvist et al (1990) following on from their experiments in obstruction alone, showed that when the obstructed area was resected and an anastomosis formed there was no difference in the collagen content in the area of the stitch line and the areas proximal and distal to it when compared to anastomoses without an obstruction. However the collagen synthesis levels proximal, but not distal, to the anastomosis were three fold higher in the obstructed group on days 0 and 2 but not on day 4 post-anastomosis. This implies that proximal to the anastomosis in the first two days there must be an increase in lysis, which is consistent with the immunolocalisation of MMPs in the same area demonstrated in this thesis.

The underlying stimulus for MMPs in the obstructed colon was compounded by performing an anastomosis as the MMPs and TIMP-1 were more widespread and persisted for longer. The distribution of the MMPs and TIMP was more exaggerated than in the normal healing model where the enzymes were restricted to the suture line. Although the damage with its associated histological and immunohistochemical changes was more pronounced than with obstruction alone, it was still relatively transient as by the seventh post-anastomosis day the damage was restricted to the stitch line, not dissimilar from the normal healing model. This demonstrates the ability of the colonic
tissue, especially the mucosal epithelial border to regenerate aided by the MMPs in degrading and remodelling of the tissue.

The oedema of the submucosa which was a marked feature of before and after resection and anastomosis in obstruction appears to be predominantly resolved by gelatinase as this was the major MMP present in this layer. The large number of cells containing gelatinase suggests that there is likely to be some extracellular enzyme present but insufficient for the resolution of the light microscope. Gelatinase is able to degrade denatured collagen so that damaged collagen fibres in this connective tissue layer is probably broken down by this enzyme and then removed by the phagocytes which have infiltrated the submucosa.

6.4 Ischaemic model

6.4.1 The ischaemic segment

Most experimental work in intestinal ischaemia focused on tissue damage due to reperfusion rather than ischaemia using animals where ischaemia was only maintained for short-periods, usually less than 6 hours. This time period of ischaemia in this work was shown to produce only superficial damage and no expression of MMPs. In developing an ischaemia model, the aim was to produce irreversible ischaemic damage with evidence of tissue necrosis in all layers. In the centre of the ischaemic segment histological damage, consistent with tissue death was found and there was no synthesis of MMPs or TIMP. The disruption of the mucosal barrier in the centre of the ischaemic segment with the loss of the epithelial border resulting in a large ulcerated surface is consistent with other ischaemic models e.g. using microspheres injected into dog colon (Boley et al 1965) or tying the vessels to the colon also in dogs (Marston et al 1969). The damage in this model was not as extensive in the strips away from the central portion where some viable tissue and MMPs were demonstrated, more frequently in the distal than in the
proximal portions of the ischaemic segment. When present the MMPs were mostly collagenase and stromelysin, extracellularly in the mucosa which histologically showed loss of normal crypt pattern and of the epithelial cell border and was the most damaged layer indicative of its vulnerability to ischaemia. Intracellular gelatinase was not a feature in the submucosa as in the obstruction models but neither was oedema as prominent in the ischaemic segment.

This paucity of enzymes and their inhibitor in areas of ischaemia suggest that oxygen is necessary for synthesis of the MMPs and TIMP as it is for collagen production (Udenfriend 1966). The laser doppler measurements demonstrated that the blood flow was absent in the centre and was diminished towards the boundaries of the ischaemic segment with normal tissue, where oxygen supply was probably sufficient in these regions especially in the distal segment to allow synthesis of the MMPs. It is possible that this is due to a preferential circulation and oxygenation in the distal portion. The relevance of oxygen levels to anastomotic healing was challenged by Kirk and Irvin (1977) who in rats demonstrated that increasing the oxygen concentration to 50% did not improve healing when compared to animals breathing air. However these studies are irrelevant as it would have been more appropriate to examine either the effect of hypoxia on anastomotic healing or the benefit of oxygen therapy in an ischaemic anastomosis.

Fibroblasts are an essential component of the healing response and MMP synthesis. With the exception of polymorphonuclear leukocytes gelatinase, MMPs appeared to be synthesised by fibroblasts. Niinikoski (1979) showed that bacteria impair intracellular collagen metabolism by parasitising available oxygen from tissue fibroblasts. The loss of the epithelium in the ischaemic segment allows infiltration of bacteria and infection which can further reduce the oxygen supply. Fibroblast proliferation is arrested below an oxygen tension of 25mmHg (Silver 1973) and so less collagen, MMPs and TIMP-1 can be synthesised. Even if fibroblasts were synthesising MMPs, the enzymes would be inactive since pH is very low in ischaemic tissue (Hunt and Pai 1972). In this
environment only cysteine proteinases are likely to be active (Murphy and Reynolds 1993).

6.4.2 Ischaemia and anastomosis

The histological changes and distribution of the MMPs and TIMP-1 at the stitch line were the same as occurred in the normal healing model (6.2). However the tissue abnormalities, similar to the obstructed colon, spread beyond this area to include the entire anastomotic segment, the distal and immediate proximal segments unlike the obstruction model where the entire colon was involved.

Outside the suture line the histological abnormalities of the mucosa were patchy loss of the epithelial border, slight haemorrhage, oedema and inflammatory cell infiltrate which were seen at 12h and 24h post-anastomosis but by the third post-anastomosis day had virtually resolved. The mucosa was the layer outside the stitch line in which extracellular MMPs predominated but unlike the obstruction model these enzymes were not restricted to this layer. The MMPs were more predominant at 24h post-anastomosis compared to 12h but at both times were restricted to those segments in which histological tissue damage had been observed. Although some TIMP-1 was seen at 12h post-anastomosis, by 24h it was consistently present at the same sites as the MMPs. By the third post-anastomosis day only intracellular MMPs and TIMP were present compatible with the minor tissue damage still present.

The deeper layers of the colon in the distal, anastomotic, and the adjacent proximal segments showed more extensive infiltration of inflammatory cells than the mucosa, especially in the submucosa. The MMPs were only present intracellularly at 12h post-anastomosis but by 24h extracellular MMPs and TIMP-1 were found in the submucosa and occasionally in the muscle with many more cells expressing intracellular
MMPs and TIMP-1. By the third day only a few cells with intracellular enzymes and TIMP-1 were present in the distal anastomotic and adjacent proximal segments.

Despite resection of all macroscopically ischaemic tissue, tissue damage and expression of MMPs and TIMP-1 was more extensive than in the normal healing model but not as extensive as in the obstruction model. If the pronounced expression of MMPs is compounded by obstruction or ischaemia this was not manifest following ischaemia until 24h post-anastomosis suggesting that there was a lag before MMP synthesis. This may reflect a different mode of stimulation.

Shandall et al (1985) used a similar model to investigate the effect of oxygen tension on anastomotic healing and found a correlation between a reduction in oxygen tension and dehiscence. The MMPs cannot be responsible for the breakdown at lowest oxygen levels as they are not synthesised; dehiscence must then be due to the cell death. However where the blood supply is compromised, relative ischaemia in the vicinity of the anastomosis may explain the pattern of MMP expression adjacent to the anastomosis. This is only speculative as the blood flow at the site of anastomosis was not measured. The incidence of minor leaks reported by Shandall et al could be due to MMPs when they are not restricted to the suture line and in the absence of their inhibitor as seen in ischaemia or obstruction where they may cause excessive degradation.

6.5 Comparison of the models

The most obvious difference between the models is the very restricted localisation of the MMPs and TIMP-1 in the normal healing model and the wider distribution in the obstruction and the ischaemia models. However at the stitch line in all models a similar pattern of MMPs occurred and by the seventh post-anastomosis day there was no difference histologically or in MMP distribution. The pattern of expression at this time
indicated a tightly controlled remodelling process which would continue at a similar level until either the normal bowel structure was restored or a mature scar had formed.

In all models in the first 12h there was very much less TIMP-1 present than the MMPs suggesting that at this time uncontrolled breakdown of tissue was occurring but from then onwards in all models TIMP-1 usually co-localised with the MMPs.

The pattern of expression in the normal healing model at the different periods of the study is indicative of a well regulated process, localised only to those areas of tissue which need to be degraded for restoration of the bowel wall structure. Whereas, in the ischaemia and the obstruction models, the MMPs are present extracellularly in areas of microscopic damage which may account for their presence. In the obstruction model intracellular enzymes were present even in microscopically normal areas which may reflect a far reaching injury caused by obstruction.

The obstruction and the ischaemia models are similar in that in both, matrix metalloproteinases occur away from the stitch line but in the obstruction model extracellular MMPs and TIMP-1 were seen only in the mucosal layer whereas in the ischaemia model they extended into the submucosa. However in the obstruction model MMPs extended, throughout the colon and not only to the segments adjacent to the anastomosis as seen in the ischaemia model. Intracellular gelatinase in the submucosa was a prominent feature of the obstruction model although it was also present to a lesser extent in the ischaemia model. With obstruction oedema in the submucosa may have caused collagen distortion which could account for the abundant presence of intracellular gelatinase. In ischaemia however, there is more destruction in the submucosal connective tissue which may demand the presence of extracellular enzymes. Furthermore in the ischaemia model, MMPs and TIMP-1 are much more pronounced 24h post-anastomosis than at 12h, whereas this difference was not seen in the obstruction model. This may indicate different stimuli for MMPs synthesis.
6.6 The interplay of MMPs and TIMP-1

6.6.1 Introduction

In all models a similar time course of expression of the MMPs and TIMP-1 was seen. TIMP-1 expression was not as pronounced until after 12h post-anastomosis and always at the same site as the MMPs for the remaining study period. Collagenase was always the most prominent enzyme in the initial phases (first 24h) although stromelysin and gelatinase were present but at later times stromelysin predominated. Where oedema and a large inflammatory cell infiltrate were present many cells containing intracellular gelatinase was the dominant feature observed most commonly in the submucosa. This feature was more obvious in obstruction than in ischaemia.

The MMPs and TIMP-1 are usually defined as either intracellular or extracellular. This distinction is important because the substrates for these enzymes are the extracellular matrix components. Whenever they are observed only inside the cells they are not active. These enzymes are not present in normal tissue therefore the presence of intracellular enzyme reflects the ability of the tissue cells to synthesise and presumably secrete the enzymes on demand. It is believed that wherever intracellular enzyme occurs there is secretion at low levels below the detection sensitivity of the light microscope. The use of a confocal microscope which allows images to be built up would resolve this difficulty. Antisera detects extracellular MMPs as pro-enzyme, as active enzyme and as enzyme-TIMP complex and these may be differentiated by certain immunohistochemical characteristics which will be described later in the relevant sections. TIMP-1 cannot bind directly to matrix or to pro-enzyme so that wherever it is seen on the matrix it must be bound to one of the active MMPs.
Collagenase was the dominant enzyme for the first 24 hours post-injury. It was seen extracellularly mainly on the mucosal matrix and intracellularly in all layers in cells with a fibroblast-like appearance. Unlike the other MMPs, pro-collagenase cannot bind to the matrix, therefore the presence of extracellular enzyme on the mucosal matrix is indicative of an active enzyme (Allan et al 1991). As TIMP-1 was scarcely present at 12 hours collagen degradation must be taking place. The initial dominance of collagenase may be a reflection of its specific action on the triple-helix portion of interstitial collagens; a role specific to this enzyme. Once collagenase has destabilised the helix, the collagen molecule becomes susceptible to degradation by many other proteases including the stromelysins and gelatinases.

Collagenase was still present at 3 and 7 days post-operatively but was less dense as most of the interstitial collagen had already been degraded and further degradation was completed by the other MMPs and phagocytes.

Intracellular collagenase was always in cells with a spindle shaped nucleus which were probably fibroblasts. Although collagenase can be synthesised and stored by polymorphonuclear leukocytes, polymorphonuclear leukocyte collagenase is immunologically distinct from interstitial collagenase (Murphy and Sellers 1980a, Weiss and Peppin 1986, Mainardi et al 1987). The antiserum used in this study has only a weak affinity for polymorphonuclear leukocytes collagenase. Thus the collagenase described in this study was interstitial collagenase not neutrophil collagenase (Hembry et al 1986).
6.6.3 Stromelysin

Stromelysin showed an inverse relationship with collagenase. There was less enzyme in the first 24 hours and increased at 3 and 7 days, although at 7 days the enzyme present was mainly intracellular. The time course observed for stromelysin may be related to its action on a broader range of substrates than collagenase including denatured collagen, proteoglycans, laminin and fibronectin. Hence collagenase initiates the degradation process and stromelysin assists in the clearing up. In the early stages an important action of stromelysin may be its ability to fully activate collagenase, increasing its activity 10-fold at a molar ratio of 1:100 (Murphy et al 1987). Cultured cells which only secrete collagenase are unable to degrade type I collagen films unless exogenous stromelysin is added (Murphy et al, 1992). The reverse also occurs in that collagenase can activate stromelysin (Alexander and Werb, 1989).

Both pro- and active stromelysin can bind to the matrix through the C-terminal domain (Allan et al, 1991) and as the antiserum detects both forms whether the enzyme is active or not cannot be determined by the immunolocalisation method used. However, since these enzymes are only produced on demand it is likely that the enzyme when present is active. Moreover collagenase and stromelysin share the same activating factors, hence the presence of active collagenase would suggest that when stromelysin is detected it is probable that it is also in an active form. As extracellular TIMP-1 only co-localises with active MMPs as already described (6.6.1.) this is further evidence that stromelysin is active.

Synthesis of stromelysin and collagenase are frequently co-ordinately regulated (Murphy and Reynolds, 1993; Girard et al 1993, Matrisian, 1990, Woessner 1991), therefore their presence at the same site is not surprising, however the dominance of one enzyme at any time suggests that other independent regulatory pathways are also involved. Our knowledge of in vivo activators of MMP synthesis is very limited at
present. From in vitro work, interleukin-1 is known to be a particularly potent activator of collagenase and stromelysin synthesis. Interleukin-1 is almost certainly present wherever MMPs are detected, released from inflammatory cells especially macrophages. Once synthesised, pro-stromelysin as well as pro-collagenase, are most likely activated by plasmin formed from plasminogen. With the amount of haemorrhage and tissue damage seen in the models examined this would be a plausible concept.

6.6.4 Gelatinase

The levels of gelatinase in the suture line were similar throughout the experimental period. In the obstruction and healing models gelatinase was also seen transiently as an extracellular enzyme away from the stitch line but more predominantly intracellularly especially in the submucosa associated with at least two different cell types mainly polymorphonuclear leukocytes but also cells with a spindle-shaped nuclei, probably fibroblasts. Gelatinases primarily degrade denatured collagens (gelatins) and type IV and V collagens. Hence their role in these models is probably to further degrade the collagen once collagenase has acted. The fragments produced are then phagocytosed by the inflammatory cells present.

Like stromelysin, both pro- and active forms of gelatinase can bind to the matrix and the antiserum used detects both forms, hence whether the enzyme present is active or not cannot be established by the immunohistochemical method used. However for some of the reasons mentioned already since these enzymes are produced on demand whenever the enzyme is present extracellularly it is probably in its active form. The co-localisation of TIMP-1 and extracellular gelatinase can only occur if the enzyme is active. Both gelatinase synthesis and activation of the pro-enzyme are regulated by different mechanisms to those of collagenase and stromelysin. Indeed some factors which stimulate the synthesis of gelatinase for example, transforming growth factor β, decrease collagenase synthesis (Edwards et al 1987, Overall et al 1989). The different pathways
for gelatinase may be related to its location on a separate chromosome to collagenase and stromelysin (Spurr et al 1988, Huhtala et al 1990).

Both pro-gelatinase A and B can be auto-activated. Pro-gelatinase A may also be activated by a fibroblast cell membrane component and gelatinase B by oxygen radicals (Peppin and Weiss 1986), but as yet the in vivo stimulation of synthesis remains unclear.

6.6.5 TIMP-1

TIMP-1 was always seen at the same location as the MMPs increasing in density with time for the first three days and then gradually diminishing. This suggests that the degradation is initially uncontrolled but as the process progresses TIMP-1 regulates the MMP activity in a co-ordinated healing mechanism. TIMP-1 only binds to active MMPs and not to the extracellular matrix components, hence where TIMP is observed on the matrix it has to be bound to active enzyme.

Synthesis of TIMP-1 is activated by the same factors which stimulate collagenase and stromelysin synthesis. Therefore their co-localisation is not surprising and this is probably a very important regulatory mechanism to prevent extensive matrix degradation. There are however notable exceptions to this co-ordinate regulation for example, transforming growth factor β stimulates TIMP synthesis but down regulates expression of collagenase and stromelysin.

6.7 Possible regulatory mechanisms

In vitro many factors are implicated in the stimulation of the synthesis and activation of pro-collagenase and pro-stromelysin but little is known of the in vivo stimulators. In the models studied, from the variety of cell types present and the tissue damage observed possible stimulatory factors can be suggested.
Firstly, local stimulation for the synthesis of MMPs may be initiated by the damaged tissue. The mechanical or chemical damage to the tissue may release cytokines such as interleukin-1 and tumour necrosis factor α (Gowen et al 1984, Dayer et al 1985, Brenner et al 1989) and growth factors such as basic fibroblast growth factor and platelet-derived growth factor (Edwards et al 1987, Bauer et al 1985, Kerr et al 1988). With the inflammatory reaction there is a rich infiltration of polymorphonuclear leukocytes into the damaged area. Polymorphonuclear leukocytes contain MMPs and are also a source of cytokines, such as interleukin-1 which stimulate MMP synthesis (Murphy and Reynolds 1985a, Pasternack et al 1986). The involvement of polymorphonuclear leukocytes in anastomotic healing in rats was shown either by eliminating them inducing neutropenia or by inhibiting their release of oxygen free radicals, of plasminogen activators and of MMPs. This prevented the normal loss of tissue strength following an anastomosis (Hogstrom and Haglund 1986, Hogstrom et al 1988, Jonsson and Hogstrom 1991). Polymorphonuclear leukocytes isolated from wounds produce significantly more superoxide than polymorphonuclear leukocytes from blood (Paty et al 1990) which suggests that the polymorphonuclear leukocytes are primed by the extravascular migration into wounds. This could be an important mechanism of activation of the MMPs in wound healing.

Secondly, changes in the normal interactions between cells and extracellular matrix are known to stimulate MMP synthesis for example, when fibronectin fragments interact with the fibronectin receptor on a cell, which occurs only in damaged tissue, this results in the induction of matrix metalloproteinases expression (Werb et al 1989). Similarly, interaction of laminin fragments with their receptor has been demonstrated to stimulate the synthesis of collagenase (Martin and Timpl 1987).
Finally cell to cell interactions may also be important, as cultures of keratinocytes which secrete interleukin-1 were found to stimulate synthesis of collagenase and stromelysin by fibroblasts (He et al 1989).

Stimulated cells secrete a pro-enzyme which is activated extracellularly. For collagenase and stromelysin the activation is probably by plasmin derived from plasminogen by the action of the plasminogen activators, urokinase (uPA) and tissue plasminogen activator (tPA) released from damaged tissue (Salonen et al 1985, Knudsen et al 1986). Further evidence for cell to cell interactions regulating MMPs came from Johnson-Wint (1980) who found that medium conditioned by epithelial cells from adult rabbit cornea was able to both inhibit and stimulate synthesis of pro-collagenase by stromal cells from the same source. Activation of gelatinase appears to be by some other mechanism, possibly involving auto-activation. For gelatinase A a fibroblast cell membrane component as yet unidentified appears to activate the pro-enzyme (G. Murphy, personal communication) and for gelatinase B oxygen radicals may be the activator (Peppin and Weiss 1986).

6.8 Conclusions

An indirect immunofluorescence technique has allowed the localisation of the MMPs and TIMP-1 in colonic tissues. This study has demonstrated that the distribution of collagenase, gelatinase and stromelysin is related to the extent and intensity of tissue damage suggesting that they have a role in colonic healing. In a colonic anastomosis the MMPs are restricted to the stitch line where the tissue has been disrupted for degradation and remodelling to occur. The presence of TIMP-1 in the same location suggests the MMPs' action is tightly controlled. In obstruction and ischaemia MMPs were widely spread in the colon and their distribution was not restricted to the stitch line as in anastomosis in a normal colon. However by seven days the immunohistochemical appearances at the anastomosis were identical.
In all models the lack of TIMP-1 in relation to MMPs, particularly collagenase, in the early period suggest that uncontrolled degradation of the matrix may initially occur. In the normal healing model a constant feature seen in this study and by Chowcat et al (1988) is the presence of TIMP at the same site as the MMPs 24h after anastomosis demonstrating a controlled remodelling process. However in obstruction and ischaemia the more widespread distribution of the MMPs and the relative absence of TIMP-1, may disturb the balance between degradation and remodelling leading to delayed healing.

The significance of these observations in relation to healing can only be affirmed by correlating the expression of MMPs with collagen concentrations. This is technically unattainable as the tissue of interest cannot be simultaneously processed for immunohistochemistry as well as biochemical analysis.

The observations in this study project an overall pattern, as any specific tissue change in space and time can be difficult to identify because of the dynamic nature of the healing mechanism following injury. The interplay of individual metalloproteinases with one another, with their inhibitor, the matrix and the cell types present demonstrate the complexity of the biochemical events that should be considered before understanding the mechanisms of healing. It is appropriate, but early, to consider modes of modulating the healing process.
REFERENCES


Halsted WS (1913). Ligature and suture material. The employment of fine silk in preference to catgut and the advantage of transfixion of tissues and vessels in control of hemorrhage. Also an account of the introduction of gloves, guta-percha tissue and silver foil. JAMA 60: 1119-1126.


