EFFECTS OF CHEMOTHERAPY ON BONE AND BONE
REGENERATION USING TISSUE ENGINEERING
TECHNIQUES

By

KUANG-SHENG LEE

SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN THE UNIVERSITY OF LONDON

NOVEMBER 2001

CENTRE FOR BIOMEDICAL ENGINEERING

INSTITUTE OF ORTHOPAEDICS AND MUSCULO-SKELETAL SCIENCE
ROYAL FREE AND UNIVERSITY COLLEGE MEDICAL SCHOOL
ROYAL NATIONAL ORTHOPAEDIC HOSPITAL
BROCKLEY HILL
STANMORE
MIDDLESEX
UNITED KINGDOM
Contents

Abstract III

Acknowledgement V

List of figures VI

List of Tables X

Chapter One An Introduction to the treatment of bone tumours and tissue engineering in bone regeneration 1

Chapter Two Effects of short exposure of fibroblast growth factor-2 on mesenchymal stem cells 62

Chapter Three Effects of chemotherapy on mesenchymal stem cells 84

Chapter Four Effects of chemotherapy on adult and immature rat skeleton 109

Chapter Five Effects of chemotherapy on bone regeneration 127

Chapter Six Use of mesenchymal stem cells to facilitate bone regeneration in normal and chemotherapy-treated rats 143

Chapter Seven General Discussion 180

References 194

Appendix I 276

Appendix II 292
Reconstructing segmental bone defects after resection of malignant bone tumours is a long-standing clinical problem. With the increased knowledge of mesenchymal stem cells (MSCs), it may now be possible to reconstruct segmental bone defects using a tissue engineering approach. Treatment of bone tumours such as osteosarcoma involves chemotherapy. These chemotherapeutic agents are potent inhibitors of cell division and these drugs may affect regeneration of bone from osteoprogenitor cells.

The ultimate aim of this study was to investigate the use of mesenchymal stem cells for repairing segmental bone defects after tumour resection. The effects of chemotherapeutic drugs on (1) differentiation of mesenchymal stem cells; (2) natural repair of bone defects; (3) regeneration and remodeling of the bones were studied.

The in vitro effects of fibroblast growth factor-2 on mesenchymal stem cells were investigated and it was found that there was time and dose-dependent effects of fibroblast growth factor-2 on proliferation and chondrogenic differentiation but not osteogenic differentiation of mesenchymal stem cells. Secondly, the toxicity of chemotherapy agents on mesenchymal stem cells was demonstrated. Cisplatin and doxorubicin significantly inhibited proliferation and osteogenic differentiation of mesenchymal stem cell. Methotrexate did not inhibit proliferation if the cells were pre-treated with osteogenic supplements. The systemic influences of these chemotherapy agents on the adult as well as the immature rat skeleton were also
quantitatively analysed. Ultimate bending (p=0.018 in adult rats and 0.061 in immature rats) and torsional strength (p=0.026 in adult rats and p=0.056 in immature rats) of the femur as well as the ultimate shear strength of the distal femur physis (p=0.044) was significantly reduced following chemotherapy. A significant reduction of cell numbers within the growth plate (p<0.001) and a change of growth plate morphology was evident. Using dual energy X-ray absorptiometry, radiography and histology it was demonstrated that bone regeneration was delayed in chemotherapy-treated rats in a femoral bone defect model. Finally, bone regeneration in normal and chemotherapy-treated rats was enhanced with mesenchymal stem cells and injectable fibrin glue scaffolds. It was shown that MSCs with fibrin glue could remain viable for up to 96 hours in tissue culture. When MSCs were used in conjunction with fibrin glue in vivo then the effects of chemotherapy could be alleviated and bone formation significantly enhanced. These studies demonstrated that the effect of systemic administration of chemotherapeutic agents on bone strength, regeneration and repair and indicated that a tissue engineering approach in patients undergoing chemotherapy may be beneficial for treating segmental bone defects after tumour resection.
ACKNOWLEDGEMENTS

I consider myself very fortunate that I can have the opportunity to join the department and have Professor Gordon Blunn as my PhD supervisor. Thank you to Professor Blunn for devoting so much of your time and effort to the success of this work. Without your help and patience, it is impossible for an overseas student like myself to finish all the work in two years.

Thank you to Professor Allen Goodship for your joint supervision and contribution to my research.

Thank you to the Ministry of Education, Taiwan for the financial sponsorship. Thank you to the Committee of Vice Chancellors and Principals, United Kingdom for awarding me an Overseas Research Student Scholarship.

The research was carried out in the Centre for Biomedical Engineering, the Institute of Orthopaedics and the Royal Veterinary College. I am deeply indebted to members of staff who have all at one point directly contributed to their time and assistance to this work. Special thanks to Annie Bartram, Mark Harrison, Keith Rayner and Mary Wait in BME, Mike Kayser in the Institute and Gillian Hughes in RVC.

I am privileged to work alongside some remarkable people and I acknowledge and am grateful for their contributions and support.

Writing this thesis took so much hard work with many obstacles to conquer. It was only through faith, belief and His grace, mercy and love that it is now complete.

*Jesus Christ is the same yesterday today and forever* *(Hebrews 13:8).*

This thesis is dedicated to my wife Catherine, my daughters Elizabeth and Isabelle. Thank you for being there and supporting me all the time.
<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Caption</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Stanmore custom-made tumour prosthesis</td>
<td>13</td>
</tr>
<tr>
<td>1-2</td>
<td>A diagram showing differentiation of human mesenchymal stem cells</td>
<td>40</td>
</tr>
<tr>
<td>2-1</td>
<td>ANOVA analysis in $^3$H-Thymidine incorporation assay of MSCs treated with different concentrations of FGF-2 at Day 2</td>
<td>72</td>
</tr>
<tr>
<td>2-2</td>
<td>ANOVA analysis in $^3$H-Thymidine incorporation assay of MSCs treated with different concentrations of FGF-2 at Day 7</td>
<td>73</td>
</tr>
<tr>
<td>2-3</td>
<td>ANOVA analysis in $^3$H-Thymidine incorporation assay of MSCs treated with different concentrations of FGF-2 at Day 14</td>
<td>73</td>
</tr>
<tr>
<td>2-4</td>
<td>ANOVA analysis in $^3$H-Thymidine incorporation assay of MSCs treated with different concentrations of FGF-2 at Day 19</td>
<td>74</td>
</tr>
<tr>
<td>2-5</td>
<td>ANOVA analysis in alkaline phosphatase expression of MSCs treated with different concentrations of FGF-2 at Day 2</td>
<td>75</td>
</tr>
<tr>
<td>2-6</td>
<td>ANOVA analysis in alkaline phosphatase expression of MSCs treated with different concentrations of FGF-2 at Day 7</td>
<td>75</td>
</tr>
<tr>
<td>2-7</td>
<td>ANOVA analysis in alkaline phosphatase expression of MSCs treated with different concentrations of FGF-2 at Day 14</td>
<td>76</td>
</tr>
<tr>
<td>2-8</td>
<td>ANOVA analysis in alkaline phosphatase expression of MSCs treated with different concentrations of FGF-2 at Day 19</td>
<td>76</td>
</tr>
<tr>
<td>2-9</td>
<td>Type 2 collagen immunostaining at Day 11 in 0.1 ng/ml group</td>
<td>77</td>
</tr>
<tr>
<td>2-10</td>
<td>Type 2 collagen immunostaining at Day 11 in 1 ng/ml group</td>
<td>77</td>
</tr>
<tr>
<td>2-11</td>
<td>Type 2 collagen immunostaining at Day 11 in 10 ng/ml group</td>
<td>78</td>
</tr>
<tr>
<td>2-12</td>
<td>Type 2 collagen immunostaining at Day 11 in 100 ng/ml group</td>
<td>78</td>
</tr>
<tr>
<td>2-13</td>
<td>Type 2 collagen immunostaining at Day 11 in the control group</td>
<td>78</td>
</tr>
<tr>
<td>2-14</td>
<td>ANOVA analysis in $^3$H-Thymidine incorporation of MSCs treated with different exposure time of FGF-2 at Day 7</td>
<td>79</td>
</tr>
<tr>
<td>3-1(A)</td>
<td>Figure 3-1 (A). SEM image of MSCs in group 1 on day 21 (Mag x200)</td>
<td>104</td>
</tr>
<tr>
<td>FIGURE LIST</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>3-1(B) Figure 3-1 (B). SEM image of MSCs in group 2 on day 21 (Mag x200)</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>3-1(C) Figure 3-1 (C). SEM image of MSCs in group 3 on day 21 (Mag x200)</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>3-1(D) Figure 3-1 (D). SEM image of MSCs in group 4 on day 21 (Mag x200)</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>4-1 Setup of material testing machine for four-point bending test</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>4-2 Testing rig for four-point bending test</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>4-3 Testing Rig for ultimate torsion test</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>4-4 Bone fractured under torsional load</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>4-5 Separation of distal femur physis under shear load</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>4-6 An example of load-deformation curve of a four-point bending test in the adult control group</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>4-7 An example of load-deformation curve in the test of ultimate shear strength of the distal femoral physis in the control group</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>4-8 An example of histology of the growth plate in the control group (Mag x100)</td>
<td>122</td>
<td></td>
</tr>
<tr>
<td>4-9 An example of the histology of the growth plate in the chemotherapy group (Mag x100)</td>
<td>122</td>
<td></td>
</tr>
<tr>
<td>5-1 A photograph showing the external skeletal fixator used in this study</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td>5-2 DEXA scanning</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>5-3 Mounting device for DEXA scan</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>5-4 A radiograph showing bone regeneration three weeks after the operation in the control group</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td>5-5 A radiograph showing bone regeneration three weeks after the operation in the chemotherapy group</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td>5-6 A radiograph showing bone regeneration five weeks after the operation in the control group</td>
<td>137</td>
<td></td>
</tr>
<tr>
<td>5-7 A radiograph showing bone regeneration five weeks after the operation in the chemotherapy group</td>
<td>137</td>
<td></td>
</tr>
<tr>
<td>FIGURE LIST</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>5-8</strong></td>
<td>Histology of the regenerated tissue in the osteotomy gap in the control group five weeks after the operation (Mag x160)</td>
<td>138</td>
</tr>
<tr>
<td><strong>5-9</strong></td>
<td>Histology of the regenerated tissue in the osteotomy gap in the chemotherapy group five weeks after the operation (Mag x160)</td>
<td>138</td>
</tr>
<tr>
<td><strong>5-10</strong></td>
<td>Bone mineral density in the osteotomy gaps of the rats treated and not treated with chemotherapy</td>
<td>139</td>
</tr>
<tr>
<td><strong>6-1</strong></td>
<td>A photograph showing lateral incision for femoral bone exposure</td>
<td>150</td>
</tr>
<tr>
<td><strong>6-2</strong></td>
<td>A photograph showing that soft tissues were removed and the femur bone was retrieved</td>
<td>150</td>
</tr>
<tr>
<td><strong>6-3</strong></td>
<td>Tisseel® kit</td>
<td>154</td>
</tr>
<tr>
<td><strong>6-4</strong></td>
<td>Injection apparatus of Tisseel®</td>
<td>154</td>
</tr>
<tr>
<td><strong>6-5</strong></td>
<td>A photograph showing the external skeletal fixator used in this study</td>
<td>158</td>
</tr>
<tr>
<td><strong>6-6</strong></td>
<td>Rat mesenchymal stem cells (Paasage 1, Mag x100)</td>
<td>159</td>
</tr>
<tr>
<td><strong>6-7</strong></td>
<td>SEM image of rat mesenchymal stem cells (Mag x330)</td>
<td>159</td>
</tr>
<tr>
<td><strong>6-8</strong></td>
<td>Alamar blue assay of MSCs in fibrin glue</td>
<td>161</td>
</tr>
<tr>
<td><strong>6-9</strong></td>
<td>(^3)H-Thymidine incorporation assay of MSCs in fibrin glue</td>
<td>161</td>
</tr>
<tr>
<td><strong>6-10</strong></td>
<td>SEM image of MSCs in fibrin glue for 24 hours (Mag x600)</td>
<td>162</td>
</tr>
<tr>
<td><strong>6-11</strong></td>
<td>SEM image of MSCs in fibrin glue for 96 hours (Mag x600)</td>
<td>162</td>
</tr>
<tr>
<td><strong>6-12</strong></td>
<td>Histology of MSCs in fibrin glue for 24 hours (Mag x 600)</td>
<td>163</td>
</tr>
<tr>
<td><strong>6-13</strong></td>
<td>Histology of MSCs in fibrin glue for 96 hours (Mag x 600)</td>
<td>163</td>
</tr>
<tr>
<td><strong>6-14</strong></td>
<td>Histology showing dividing MSCs (Mag x 600)</td>
<td>163</td>
</tr>
<tr>
<td><strong>6-15</strong></td>
<td>TEM image of MSCs in fibrin glue for 24 hours (Mag x20,000)</td>
<td>164</td>
</tr>
<tr>
<td><strong>6-16</strong></td>
<td>TEM image of MSCs in fibrin glue for 96 hours (Mag x20,000)</td>
<td>164</td>
</tr>
<tr>
<td><strong>6-17</strong></td>
<td>Radiograph showing bone regeneration in Group 1 (Mag x20)</td>
<td>168</td>
</tr>
<tr>
<td>FIGURE</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>6-18</td>
<td>A radiograph showing bone regeneration in Group 2 (Mag x20)</td>
<td>168</td>
</tr>
<tr>
<td>6-19</td>
<td>A radiograph showing bone regeneration in Group 3 (Mag x20)</td>
<td>169</td>
</tr>
<tr>
<td>6-20</td>
<td>A radiograph showing bone regeneration in Group 4 (Mag x20)</td>
<td>169</td>
</tr>
<tr>
<td>6-21</td>
<td>A radiograph showing bone regeneration in Group 5 (Mag x20)</td>
<td>170</td>
</tr>
<tr>
<td>6-22</td>
<td>A radiograph showing bone regeneration in Group 6 (Mag x20)</td>
<td>170</td>
</tr>
<tr>
<td>6-23</td>
<td>ANOVA analysis of DEXA scan results (g/cm^3) at the osteotomy gap three weeks after the operation</td>
<td>171</td>
</tr>
<tr>
<td>6-24</td>
<td>ANOVA analysis of DEXA scan results (g/cm^3) at the osteotomy gap five weeks after the operation</td>
<td>171</td>
</tr>
<tr>
<td>6-25</td>
<td>A photomicrograph showing bone regeneration in Group 1 (Mag x100)</td>
<td>172</td>
</tr>
<tr>
<td>6-26</td>
<td>A photomicrograph showing bone regeneration in Group 2 (Mag x100)</td>
<td>172</td>
</tr>
<tr>
<td>6-27</td>
<td>A photomicrograph showing bone regeneration in Group 3 (Mag x100)</td>
<td>173</td>
</tr>
<tr>
<td>6-28</td>
<td>A photomicrograph showing bone regeneration in Group 4 (Mag x100)</td>
<td>173</td>
</tr>
<tr>
<td>6-29</td>
<td>A photomicrograph showing bone regeneration in Group 5 (Mag x100)</td>
<td>174</td>
</tr>
<tr>
<td>6-30</td>
<td>A photomicrograph showing bone regeneration in Group 6 (Mag x100)</td>
<td>174</td>
</tr>
<tr>
<td>6-31</td>
<td>Quantitative histological analysis of percentage bone formation at the osteotomy gap</td>
<td>175</td>
</tr>
<tr>
<td>Table No.</td>
<td>Caption</td>
<td>Page No.</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>3-1</td>
<td>Experimental groups</td>
<td>95</td>
</tr>
<tr>
<td>3-2</td>
<td>Results of MTT assay in Groups 1-4</td>
<td>100</td>
</tr>
<tr>
<td>3-3</td>
<td>Results of alamar blue assay in Groups 1-4</td>
<td>101</td>
</tr>
<tr>
<td>3-4</td>
<td>Results of alamar blue assay in Groups 5-11</td>
<td>102</td>
</tr>
<tr>
<td>3-5</td>
<td>Results of alkaline phosphatase assay in Groups 1-4</td>
<td>103</td>
</tr>
<tr>
<td>4-1</td>
<td>Body weight of the rats at the time of sacrifice (g)</td>
<td>121</td>
</tr>
<tr>
<td>4-2</td>
<td>Ultimate bending strength (N)</td>
<td>121</td>
</tr>
<tr>
<td>4-3</td>
<td>Ultimate torsional strength (N)</td>
<td>121</td>
</tr>
<tr>
<td>4-4</td>
<td>Ultimate shear strength of distal femoral physis (N)</td>
<td>121</td>
</tr>
<tr>
<td>4-5</td>
<td>Cell numbers per column in the growth plate</td>
<td>121</td>
</tr>
<tr>
<td>6-1</td>
<td>Body weight of the rats at the time of sacrifice</td>
<td>167</td>
</tr>
</tbody>
</table>
Chapter One

AN INTRODUCTION TO THE TREATMENT OF BONE TUMOURS AND TISSUE ENGINEERING IN BONE REGENERATION
1.1 Limb Salvage for treatment of bone cancer

1.2 Fixation of hydroxyapatite-coating cementless bone tumour implants

1.3 Chemotherapy for malignant bone tumours

1.4 Tissue engineering in bone regeneration
   1.4.1 What is tissue engineering?
   1.4.2 Mesenchymal stem cells
   1.4.3 Scaffolds for bone tissue engineering
   1.4.4 Growth factors

1.5 Study design
The aims of the thesis are to investigate the hypotheses that:

(1) Chemotherapy drugs, at their therapeutic concentrations, are detrimental to the proliferation and osteogenic differentiation of mesenchymal stem cells.

(2) Chemotherapeutic agents influence the mechanical properties of the skeletal tissues.

(3) Chemotherapy treatment slows down the rate of bone regeneration.

(4) Use of mesenchymal stem cell-based tissue engineering approach facilitates bone regeneration when chemotherapy agents are systemically administered.

The rationale for developing the hypotheses will be introduced in the following sections. Essentially, the motive of this thesis stems from the observation of a clinical dilemma – reconstruction of bone defects after removal of bone in patients with skeletal neoplasm of the extremities. It is hoped that at the end of this thesis, a rationalised tissue engineering approach can be developed.
CHAPTER 1

1.1 LIMB SALVAGE FOR TREATMENT OF BONE CANCER

Skeletal neoplasm is a rare but difficult disease (Simon 1991). Historically, the only treatment of skeletal neoplasm of the extremities was amputation of the limb. With the advances in the treatment of bone sarcoma, it was then realised that not all bone cancers need an amputation. Preservation of the involved limb after surgical removal of the tumour has been performed for more than a century for bone sarcomas of low and moderate grade (Simon 1988). In the past two decades, advances in adjuvant and neo-adjuvant chemotherapy treatment, in diagnostic imaging and in the surgical technique for reconstruction of the limbs have led to the performance of limb salvage surgery for most patients who have high-grade sarcomas (Choong and Sim 1997).

It is important to understand the significance of grading and staging of bone tumours because this relates to prognosis. Grading of the bone tumours means the degree of malignancy of the cancer cells while staging of the bone tumours refers to the extent of involvement of the tumour (Bolling and Beauchamp 1999). Most important of all, staging and grading are important indictors of prognosis (Bentzen 2001). The higher the histological grading, the more malignant the tumour cells. The most extensively used surgical staging system for musculoskeletal sarcomas is the Enneking staging system (Enneking 1980, Enneking 1986). The higher the staging, the more difficult
surgical reconstruction will be because higher surgical grade implies greater involvement of the tumours and results in wider surgical margins.

The difficulty of limb salvage not only lies in the size of the bone defects created after surgical removal of the tumours, but also in the locations of the tumours, and in the age of the patients (Nichter and Menendez 1993). For example, the most common sites for osteosarcoma, the most common primary malignant bone tumours, are the distal femur and proximal tibia (Jaffe 1991). Therefore, the distal femoral and proximal tibial articular surfaces often have to be removed and reconstruction of a painless, free-mobile joint which is durable is challenging (Lindell and Carroll 1993).

A large number of patients with malignant bone tumours are skeletally immature (Finn and Simon 1991). Removal of the growth plates of the involved limb will result in limb length discrepancy as the patient grows and this has to be addressed (Frassica 1997).

When considering whether surgery for limb-salvage is justified, it is usual to consider long-term oncological results after limb salvage and compare them with the historical results after amputation. Comparison can be made in four broad areas: (1) the overall survival of patients; (2) the early and late morbidity for each type of reconstruction; (3)
CHAPTER 1

the function of the salvaged limb and its maintenance over a prolonged period of
follow-up and (4) the quality of life in patients (Cannon 1997).

Various methods have been developed for limb salvage reconstruction after resection
of bone tumours. Arthrodesis, particularly for tumours around the knee, has
previously been used. Although the knee joint is not reconstructed, the function of the
lower extremity can be reasonably preserved. Vascularised or non-vascularised
autologous bone graft, usually part of the fibula, or allograft, is used to reconstruct the
bone stock. Although some successful long-term results have been reported (Salai
1997), non-union, graft fracture, failure of fixation of the graft-host bone junction and
infection are the main problems associated with massive bone grafts (Scarborough
and Helmstedter 1997). Leg length discrepancy, which occurs in skeletally immature
patients, cannot be compensated by arthrodesis. Consequently, arthrodesis of the knee
joint should only be used in selected cases nowadays.

Osteoarticular segmental allograft reconstruction of the resected bone segments is
another method of reconstruction which has been used previously. The advantage of
this method is that articular surface cartilage is replaced and long-term success has
been reported using osteoarticular allograft reconstruction (Bohm 1996). It was also
reported, however, that although osteoarticular allografting is an alternative to amputation, it presents unsolved immunologic and preservation problems which make the prognosis unpredictable (Alho 1991). Scintigraphic results also showed that it takes a long time for revascularisation of the osteoarticular allograft (Bar-Sever 1997).

The risk of graft fracture can not be eliminated and long-term durability of the graft is still doubtful (Rodl 2000). It has been reported that although the current results of this technique are adequate, they are imperfect, and research should be directed at improving the outcome (Mankin 1996). Instability, degeneration, or a fracture near the articular surface of the graft are the complications of osteoarticular allografts and subsequent total knee arthroplasty are sometimes necessary as salvage procedures (DeGroot and Mankin 2000).

Distraction osteogenesis has also been used for reconstruction of bone defects in limb salvage surgery. It has been reported that distraction osteogenesis is useful in limb salvage surgery for reconstruction of bone defects (Tsuchiya 1997). However, distraction osteogenesis can only be used in patients whose articular surfaces can be preserved because it is impossible to regenerate articular cartilage simply by mechanical distraction. Hence, the application of this technique is limited and it can only be used on selected patients. In addition, bone tumour patients, because of the
nature of their disease, require almost immediate rehabilitation to maximise their quality of life. Due to size of the defects and the complicated technique as well as patient requirement, distraction osteogenesis is often not suitable for bone tumour patients.

Extracorporeal irradiation of the resected bone segment which contained tumour with very high dose irradiation and then re-implanting the segment has also been reported as a successful method for reconstruction in limb salvage surgery. This technique was first described by a group of Belgian surgeons (Uyttendaele 1988). Subsequently, some clinical success in using this technique has been reported (Araki 1999, Hong 2001). Extracorporeal irradiated autograft can be used as an alternative in the countries where allografts and prostheses are not easily available. However, there are several disadvantages in using irradiated bones for reconstruction. If the resected bone segment which contained tumour is irradiated and re-implanted, it is impossible to perform a thorough pathological examination of the tumour. The information obtained from pathological examination is important for deciding post-operative adjuvant chemotherapy regime and for predicting the prognosis. Besides, the mechanical properties of the bones will be influenced after high-dose irradiation and the strength of the irradiated bones will be reduced (Currey 1997). In a study using
technetium-99m-MDP scintigraphy to evaluate the vascularisation and integration of the irradiated autografts, it is found that revascularisation and partial bone ingrowth did not lead to a lower complication rate (Van Laere 1998). Finally, animal experiment showed that articular cartilage underwent degeneration after extracorporeal irradiation (Sabo 2000).

Autoclaving (Smith and Struhl 1988) and microwave heating (Liebergall 1998) of the bone segments have also been used. The disadvantages of autoclaving and microwave heating are similar to extracorporeal irradiation. The heat emitted during autoclaving and microwave heating would further damage the autografts (Liebergall 2000). Again, these techniques should only be used when there are no alternatives.

Allograft-prosthesis composite (APC) has also been used for reconstruction, particularly, of knee joints (Harris 1994). Compared to other techniques, APC arthroplasty has many advantages, including restoration of bone stock, customisation with conventional implant components, soft tissue attachment of tendons and ligaments, and preservation of the medullary canal of the host bone. The disadvantages of this technique include slow healing in the presence of chemotherapy, the possibility of disease transmission, and availability (Hejna and Gitelis 1997).
Besides, in a carefully performed sequential cohort study, the results of this technique seem to be inferior to massive prosthetic replacement and this comparative study suggested that limb salvage surgery using a tumour prosthesis has a better and more predictable outcome (Wunder 2001).

Reconstruction with massive prosthesis has a number of advantages, including a relatively simple technique, the modularity the prosthesis offered, immediate function, and, no worry of disease transmission. (Morris 1997). In the Royal National Orthopaedic Hospital Trust, one of the two centres in the United Kingdom that treat bone tumour patients, custom-made massive prostheses have been used for reconstruction of bone defects for more than two decades. The results of the first 218 cases of distal femoral custom-made massive prostheses for limb salvage (Unwin 1993) revealed that the overall probability of one of these early prostheses surviving for 10 years was 65%, and for 20 years it was 53%. For cases of bone tumour, the probability of surviving for 10 years was 68% (not including recurrence), for primary knee replacement cases it was 32%, for other bony diseases plus trauma it was 86%, and for cases of revised massive prostheses it was 53%. It was concluded that this type of distal femoral arthroplasty provided good medium-term results, but showed some deficiencies in the long term, particularly in younger patients. In this series,
cement was used for implant fixation. The overall loosening rate was 5% in an average follow-up period of 58 months. The results of Stanmore extendible endoprostheses for the skeletally immature also revealed that aseptic loosening was the major cause of implant failure (Unwin 1996a). Aseptic loosening was identified as the predominant cause of implant related failure in a retrospective study of a consecutive series of 168 Stanmore custom made extendible endoprosthetic replacements used in skeletally immature patients. Most of the replacements were used in the treatment of bone tumor and the remainder for the revision of failed massive endoprosthetic replacements. Since the first Stanmore extendible endoprosthesis was inserted in 1976, four types of extension mechanisms have been used. Thirty-eight of the 164 cases with follow-up data were revised, of which 19 were as a result of aseptic loosening. Survival analysis revealed that the overall probability of surviving an implant related failure was $0.512 (+/- 0.005)$ at 5 years, highlighting the high complication rate of these extendible replacements that required a revision procedure. Sixteen of the 19 aseptic loosening cases were distal femoral replacements. The probability of a patient with a distal femoral replacement surviving aseptic loosening was $0.773 (+/- 0.008)$ at 5 years.

In a large series of follow-up of 1,001 custom-made Stanmore tumour prostheses
(Unwin 1996b), it was found that the probability of a patient surviving aseptic loosening for 120 months was 93.8% for a proximal femoral replacement, 67.4% for a distal femoral prosthesis and 58% for a proximal tibial implant. In patients with distal femoral replacements the age of the patient at the time of operation and the percentage of bone resected were related to the risk of aseptic loosening. Young patients with distal femoral prostheses in whom a high percentage of the femur had been replaced had the poorest prognosis for survival. The percentage of bone removed had a significant effect in the proximal tibial replacement group, but the age of the patient did not. By contrast, neither the age nor the percentage of bone removed was a factor after proximal femoral replacement.

To combat the high incidence of aseptic loosening for young patients and for patients with failed implants after resection for bone tumors, intramedullary cementless fixation of massive tumor implants was investigated (Blunn 2000). These implants consist of a hydroxyapatite coated titanium stem (Figure 1-1). To date, 47 of these prostheses have been inserted for the treatment of primary bone tumors. Radiographs indicate that the stems are osseointegrated. Radiolucent lines have not been seen between the implant and the bone. Bone remodelling changes have been observed. In several cases in which the implant was not seated properly on the transection site,
CHAPTER 1

Bone grew to the shoulder of the implant. Bone remodelling was particularly evident in stems that were coated over their entire surface. In these cases, the implant induced local bone resorption so that the bone around the mid-stem region became thinner, with resorption of cortical bone on the periosteal surface and maintenance of bone on the endosteal surface adjacent to the stem. This effect was attributed to stress shielding, and a three-dimensional finite element model using loading data obtained from a telemetry study indicated that, where the stem was bonded to the bone over the entire surface, stresses in the outer cortex became reduced. In the finite element model, reducing the region of hydroxyapatite coating to approximately 1/3 of the stem length reduced the extent of the low-stress area in the outer cortex. Subsequently, prostheses have been coated with hydroxyapatite over only approximately 1/3 of their stem. This method of fixing the massive endoprosthesis to the bone is thought to be successful in the short-term and offers an alternative to cemented fixation.

Figure 1-1. Stanmore custom-made tumour prosthesis.
1.2 FIXATION OF HYDROXYAPATITE-COATING CEMENTLESS BONE TUMOUR IMPLANTS

A prerequisite for any orthopaedic arthroplasty or implant is permanent fixation to the surrounding skeleton with no intervening soft tissue (Sun 2001). A successful fixation should be generated quickly, as soon as possible after surgery. It should be strong, able to transmit everyday loading activities and it should exhibit life long stability. Fixation takes place by osseointegration, which was first described by Branemark (1983) as the intimate contact between a titanium implant interface and the surrounding bone. The current accepted definition for osseointegration is “contact established between normal and remodelled bone and an implant surface without the interposition of non-bone or connective tissue, at the microscopic level” (Mentag 1986).

Prostheses have been implanted into the human body by either cemented or cementless fixation methods. Although the traditional cemented fixation using polymethylmethacrylate (PMMA) bone cement can obtain immediate stability between the implant and bone, this type of prosthesis is not suitable for young, active patients in whom more stable fixation and bone growth are needed. Problems of cell necrosis from the exothermic reaction caused by the heat emitted during polymerization of bone cement, fatigue failure of cement mantle and loss of endosteal
bone are still a concern when using the intramedullary cement (Galante and Jacobs 1992). Thus, cementless fixation, primarily by biological means whereby press-fit insertion is followed by bone growth into a porous surface, has been developed. However, there is little histological evidence of sufficient bone ingrowth in retrieved uncemented porous-coated prostheses (Pidhorz 1993, Bloebaum 1997) and it has been shown that bone must be within 50 μm of the porous coating for ingrowth to occur (Bloebaum 1994). Meanwhile, fibrous rather than bony ingrowth into porous surfaces has been found, and the loss of endosteal bone still exists (Bloebaum 1994).

Bioactive materials such as HA and bioactive glass can stimulate a direct bond to form between the implant and the surrounding bone and improve osseointegration (Geesink 1987). This bone-implant bonding is one of the most important factors for implant fixation and function. HA coatings have been shown to achieve a very strong bond with living bone, even under loaded conditions and with the presence of a gap (Soballe 1991, Soballe 1992, Soballe 1993).

Cementless fixation with HA coating in Stanmore custom-made tumour prosthesis has gained encouraging short-term success (Blunn 2000) and it is the current design of choice for Stanmore prostheses. Plasma-spray HA coatings have been used as surface
coatings on metallic implants in orthopaedics since the mid-1980’s (Geesink 1989, Furlong and Osborn 1991). The advantages that are sought in this application include (1) more rapid fixation and (2) increased and more uniform bone ingrowth and/or ongrowth at the bone-implant interface (Geesink 1988, Stephenson 1991, Cook 1992). Most clinical experiences with either weight-bearing or non-weight-bearing models have shown promising results shortly after the implantation and continued fixation for up to 10 years (Capello 1997, Donnelly 1997, Magyar 1997, Neilssen 1998).

What is more exciting is that HA coating can enhance bone growth across a gap of one mm between the bone and the implant in both stable and unstable mechanical conditions, and it is capable of limiting the formation of any fibrous membrane and converting a motion-induced fibrous membrane into a bony anchorage (Soballe 1991, Soballe 1992, Soballe 1993). It has also been suggested that HA coatings have sealed the bone-implant interface, preventing migration of polyethylene particles which may reduce the incidence of osteolysis and the subsequent implant failure (Rahbek 1996, Soballe and Overgaard 1996, Coathup 2001).

Cementless fixation with hydroxyapatite-coated implants is dependent on the osseointegration at the bone-implant interface and osseointegration relies on
recruitment, proliferation, differentiation and matrix production of bone cells and osteoprogenitor precursor cells (Ohgushi and Caplan 1999). Since chemotherapy has become standard treatment for malignant bone tumours, the influences of chemotherapy on osteoprogenitor cells should be taken into consideration in terms of longevity and stability of implant fixation of the massive tumour prostheses. It is reported that cisplatin, one of the most commonly used chemotherapy drugs for malignant bone tumours (Souhami 1997), caused a temporary delay in the formation of new bone around the prosthesis in a canine diaphyseal segment replacement model (Young 1997). The effects of chemotherapy on osteoprogenitor cells, i.e., mesenchymal stem cells (MSCs) which are contained in bone marrow, remain unclear. It would be valuable to elucidate the consequences of chemotherapy on MSCs.

Besides hydroxyapatite coating of the intramedullary stem, there is also a hydroxyapatite-coating “collar” design in Stanmore MARK 5 custom-made tumour prostheses (Blunn 2000). The purpose of adding a hydroxyapatite-coating at the junction of the prosthesis adjacent to the transection site is to induce extracortical bone bridging and to increase implant fixation. This has been shown to significantly reduce the incidence of aseptic loosening. However, bony bridging occurs in only 70% of patients and often in these patients the amount of bone ingrowth into the
CHAPTER 1

collar is limited. These implants are implanted into patients who are receiving neoadjuvant chemotherapy and this may affect bone formation. So, whether and to what extent chemotherapy affects bone formation is an important question.
1.3 CHEMOTHERAPY FOR MALIGNANT BONE TUMOURS

It has been proved that chemotherapy substantially improves the disease-free and overall survival rates in patients with malignant bone tumours (Eilber 1987). In a prospective, randomized trial (Link 1986), after undergoing surgery, 36 patients were randomly assigned to a group receiving adjuvant chemotherapy or a group without adjuvant treatment. At two years the actuarial relapse-free survival was 17 percent in the control group, similar to that found in studies before 1970 (Miser 1985), and 66 percent in the adjuvant-chemotherapy group (P less than 0.001). Similar results were observed among 77 additional patients who declined to undergo randomisation but who elected observation or chemotherapy. The authors concluded that adjuvant chemotherapy increases the chances of relapse-free survival of patients with high-grade osteosarcoma, and recommended that it should be given to all such patients.

In order to assess the optimum duration of treatment and the relative contributions of the constituent drugs in randomised trials, the European Osteosarcoma Intergroup (EOI) was formed in 1982 to carry out randomised studies of sufficient size to allow investigation of important features of treatment (Souhami 1997). In the first EOI trial, the use of cisplatin and doxorubicin was investigated as this regime has been proved
to be effective as an adjuvant chemotherapy regime for osteosarcoma (Ettinger 1986).

Three hundred and seven patients with osteosarcoma were randomly assigned one or two regimens of chemotherapy (Bramwell 1992). A regimen of cisplatin and doxorubicin, given before and after surgery for a total of six cycles to patients with operable non-metastatic osteosarcoma, produced 5-year survival of 64% and progression free of 57%. These results were similar with those of trials (Link 1986, Winkler 1984) in which chemotherapy was based on the TIO regimen (Rosen 1982).

The TIO regime, which had been the basis of previous chemotherapy regimes, was a combination of seven chemotherapeutic drugs given over a period of 44 weeks. A report of the results of TIO regimen showed that 65% of 279 patients were alive and free of disease at 8 years. However, non-randomised studies of treatment did not allow unbiased comparison of outcomes (Souhami 1997) and the EOI undertook a formal comparison of a 44-week multi-drug TIO-based regimen with the 18-week cisplatin and doxorubicin two-drug regimen. The results showed that in the 407 randomised patients, 391 were eligible and have been followed up for at least 4 years.

Toxic effects were qualitatively similar with the two regimens. However, 188 (94%) of 199 patients completed the six cycles of two-drug treatment, whereas only 97 (51%) of 192 completed 18 or more of the 20 cycles of the multi-drug regimen. The proportion showing a good histopathological response (>90% tumour necrosis) to
preoperative chemotherapy was about 29% with both regimens and was strongly predictive of survival. Overall survival was 65% at 3 years and 55% at 5 years in both groups. It was found that there was no difference in survival between the two-drug and multi-drug regimens in operable, non-metastatic osteosarcoma. The two-drug regimen is shorter in duration and better tolerated, and is therefore the preferred treatment (Souhami 1997).

In Europe the two-drug regimen with cisplatin and doxorubicin has become the gold standard for treatment of malignant bone tumours such as osteosarcoma. It is therefore important to understand the toxicity that this regimen causes in the skeletal tissues of the patients who receive the treatment. Because of the systemic administration of these drugs, normal skeletal tissues are also exposed and it is not difficult to imagine that normal skeletal tissues and cells are also affected. However, the toxic effects of combined cisplatin and doxorubicin use on skeletal tissues have not previously been studied. Therefore, it is valuable to quantitatively assess the detrimental effects of the two-drug regimen on skeletal tissues as well as mesenchymal stem cells (MSCs). MSCs play an important role in achieving bone ingrowth around the cementless implants. It is important to quantitatively assess the influences of cisplatin and doxorubicin on bone regeneration. A prerequisite
of new tissue engineering therapies to treat segmental bone defects is healthy and viable MSCs or bone cells. This may be an extremely valuable tool but the effectiveness of these new techniques needs to be investigated in realistic conditions where chemotherapy is administered.
1.4 TISSUE ENGINEERING IN BONE REGENERATION

1.4.1 WHAT IS TISSUE ENGINEERING?

In order to create tissues and organs for replacement, understanding the processes of repair and regeneration is essential. The regeneration and repair of tissues are fundamentally different processes (Caplan and Goldberg 1999). Regeneration of tissues readily occurs in embryos, is almost absent in neonates (although some regenerative events have been reported), and is never observed in adults (Whitby and Ferguson 1991). Regeneration is a relatively slow process that seems to recapitulate many but not all the steps that occur in the embryos. In contrast, repair is rapid and has been evolutionarily selected to minimise the animal's vulnerability, that is, to get the animal away from danger as soon as possible (Armstrong and Ferguson 1995). Likewise, the initial steps of recovery from surgical events or wounding involve an acute inflammatory response and a sealing off of the repair site, to minimise the spread of bacteria or fungal contamination of the wound site, and to provide the rapid fill of that tissue deficit with fibrous or bridging tissue (Thornton 1968). Later, after the animal is safe, slower turnover process occurs to attempt to remodel the tissue into its natural state (Caplan and Goldberg 1999). Regeneration of a tissue re-establishes the age dependent turnover dynamics and can include the capacity of that regenerated tissue to continue to grow. Repair, however, often does not allow a continuation of
growth and provides tissue with only short-term benefit.

It was not until the late 80’s that tissue engineering was regarded as an independent branch of science. The term tissue engineering was initially defined by the attendees of the first National Science Foundation of the United States sponsored meeting in 1988 as “application of the principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationship in normal and pathologic mammalian tissues and the development of biological substitutes for the repair and regeneration of tissue or organ function” (Chapek 2000). In 1993, Langer and Vacanti summarized the early development in this field and defined tissue engineering as “an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissues or organ function (Langer and Vacanti 1993). The exercise of interdigitating these different functional talents into a coherent device has produced the working definition of tissue engineering (Brekke and Toth 1998): “Tissue engineering is an art and science by which synthetic compounds are manipulated into anatomically and/or functionally specific architectures and, when required, may be integrated with biologically active agents and/or living cells such that resultant properties of the whole are precisely suited to support the specific cell life prescribed
for recipient tissues”. Consequently, tissue engineering has now emerged as a potential alternative to tissue or organ transplantation. With this technology, tissue loss or organ failure can be treated either by implantation of an engineered biological substitute or alternatively with ex vivo perfusion systems. The tissue-engineered products may be fully functional at the time of treatment (e.g., liver assist devices, encapsulated pancreatic islets), or have potential to integrate and form the expected functional tissue upon implantation (e.g., chondrocytes embedded in a matrix carrier). In some cases, biomaterials are modified to enhance migration and attachment of the specific cell populations, which repair or replace the damaged tissue (Chapekar 2000).

As the definitions suggest, cells are a key to tissue regeneration and repair due to their proliferation and differentiation, cell-to-cell signalling, biomolecule production, and formation of extracellular matrix. The most fundamental and crucial reason is that it is still impossible to create one single living cell from lifeless materials up to now. The functionality of an engineered tissue may be structural (e.g., bone, cartilage, skin), or metabolic (e.g., liver), or both. Cells may be part of an engineered tissue or alternatively, these cells may be recruited in vivo with the help of biomaterials and/or biomolecules. When selecting the cellular component of an engineered product, it is important to identify appropriate cells and to be able to isolate them from the primary
source. Relatively easy access of the cell source(s) and abundant supply of target cells are other considerations. In addition, expansion of these cells without permanently altering the phenotype and function during the expansion phase and without introduction of any adventitious and species-specific bacterial/viral agents poses significant challenges. Finally, when genetically modified cells are used in a tissue-engineered product, there are additional concerns such as cell transformation by the vector, vector stability, and optimal function of the inserted gene (Chapekar 1996).

Scaffolds are also indispensable in tissue engineering. Actually, tissue-engineering devices can be looked upon as a three dimensional (3-D) in vivo cell culture system designed to perform a plethora of vital functions required for producing a directed host response (Brekke and Toth 1998). Both engineering and biologic issues must be taken into consideration in order to maximise the function of the tissue-engineered constructs in vivo. The gross mechanical properties of the construct, structural qualities of its internal fabrics and its 3-D architectural geometry are important. Retention of mechanical characteristics after implantation and possession of a 3-D internal geometry enable these devices to maintain a tissue void, or a space, of the prescribed size and shape for future tissue occupancy. This is especially important in tissue engineering of bone tissues because it has been known for more than five
decades that bone will grow into an adjacent tissue void if that space can be maintained and protected from encroachment by non-osseous tissue and competing cell types (Hurley 1959; Linde 1993; Levy 1994). This is known as guided bone regeneration.

A scaffold’s internal, 3-D geometry links its engineering properties to those qualities impacting host response. Architecture defines a unit’s internal spatial arrangements which, in turn, contribute substantially to its mechanical characteristic limits. By governing the scaffold’s internal spatial arrangements, the architecture design also determines quantity and shape of substratum surfaces available for colonization by in vitro cell seeding or endogenous cell populations. A construct’s apparent density, number and size of internal chambers and total void volume determine the size of individual cells and the cell population densities that the unit can accommodate (Brekke and Toth 1998). Most important of all, the scaffolds must allow the delivery of morphogenetic and regulatory proteins and peptides (Boden 1995; Hollinger 1996a). Moreover, incorporation of signal peptides into the material has been attempted to effectively mimic the extracellular matrix and induce cell migration (Grzesiak 1997; Rowley 1999). The mechanical strength of the scaffold material needs to mimic the mechanical properties of the tissue it is intended to repair or
replace. Moreover, material porosity as well as pore size distribution and continuity
greatly influences the attachment of specific cell types and interaction of the
biomaterials with the host. It is also preferable that the biomaterial degrades in vivo to
minimise the long-term biocompatibility concerns, with the material degradation rate
matching the regeneration rate of the tissue. The resulting degradation products must
be non-toxic to the host. Lastly, vascularisation of the tissue-engineered is critical for
the three-dimensional constructs greater than 1mm$^3$ to meet their nutritional and
metabolic requirements (Shea 1999).

Besides cells and scaffolds, cell signalling is also crucial in the tissue engineering
constructs in order to guide the cells in the scaffold to grow and/or differentiate into
desired cell/tissue types. The best known biomolecules are a group of proteins from
transforming growth factor- $\beta$ (TGF- $\beta$ ) superfamily called bone morphogenetic
proteins (BMPs) (Urist 1965; Urist and Strates 1971). There are several strategies for
delivery of osteoinductive growth factors (Boden 1999). First, extracted human
(Johnson 1988; Johnson 1992; Teixeira and Urist 1998) or animal growth factors
(Sciadini 1997a; Sciadini 1997b) can be delivered with scaffolds or direct injection.
Using recombinant DNA technology, recombinant osteoinductive proteins can be
fabricated, such as rhBMP-2 (Wang 1990; Yamaguchi 1991) and rhBMP-7, also
CHAPTER 1
called osteogenic protein-1, or OP-1 (Asahina 1993; Sampath 1992). The third
strategy for delivery of cell signalling biomolecules in bone tissue engineering is the
use of gene therapy. The concept involves the delivery of the gene or cDNA sequence
for an osteoinductive factor rather than the delivery of the factor itself (Fang 1996;
Riew 1998). Again, various safety and efficacy issues remain to be explored. The
choice of specific molecules, the correct dose, timing, and sequence of administration,
whether singly or in combination, still must be defined for each application and for
the specific mechanical and biological requirements. The development of optimal
delivery methods for these molecules is another important question to answer.

1.4.2 MESENCHYMAL STEM CELLS
The middle embryonic layer, the mesoderm, gives rise to all of the body’s skeletal
elements. The term mesenchyme is derived from the Greek meaning “middle (meso)
infusion” and refers to the ability of mesenchymatous cells to spread and migrate in
early embryonic development between the ectodermal and endodermal layers. It is
generally agreed that in the mesoderm of an embryo a mesenchymal stem cell is a
pleuripotent progenitor cell which divides many times and whose progeny eventually
gives rise to skeletal tissues such as bone, cartilage, tendon and ligament. By
definition, these cells are not governed by or limited to a fixed number of mitotic
divisions (Caplan 1991). These cells have the capacity for extensive replication
without differentiation, and they possess a multilineage development potential
(Bruder and Fox 1999). Recently, pleuripotent stem cells have been cultured from
human foetal tissue and have shown the ability to give rise to a variety of cell types
found in embryonic germ layers (Shamblott 1998; Thomson 1998). Many adult
tissues contain populations of stem cells that have the capacity for renewal after
trauma, disease or ageing. The cells may be found within the tissues or in other tissues
that serve as stem cell reservoirs. For example, although bone marrow is the major
source of adult haematopoietic stem cells (HSCs) that renew circulating blood
elements, these cells can be found in other tissues, such as peripheral blood, as well.
The adult bone marrow also contains mesenchymal stem cells (MSCs), which
contribute to the regeneration of different mesenchymal tissues such as bone, cartilage,
muscle, ligament, tendon and adipose tissues by various investigators (Beresford 1989;
Haynesworth 1992b; Wakitani 1994; Bruder 1998a; Yoo 1998; Johnstone 1999). In
vitro and animal studies have indicated that there is either a multipotent MSC or the
populations are the mixtures of committed progenitor cells, each with restricted
potential (Wakitani 1995; Bergman 1996; Cassiede 1996; Young 1998; Awad 1999;
Petite 2000). Recently, multi-potential human mesenchymal stem cells (hMSCs),
which were derived from bone marrow, have been well characterised. Under proper
manipulation, these hMSCs from the same donors could become osteoblasts, chondrocytes and adipocytes (Pittenger 1999).

As these MSCs are harvested from bone marrow and bone marrow is easily obtained, the understanding of bone marrow, in particular, the stromal cell system, is important. There has been an increasing interest in recent years in the stromal cell system, which includes the marrow-derived stromal cell that supports haematopoiesis, as well as the mesenchymal stem cell and its progeny, connective tissue cells such as osteoblasts, chondrocytes, tenocytes, adipocytes and smooth muscle cells. This was first described by Owen (1985). Essentially, there are three main cellular systems in the bone marrow: haematopoietic, endothelial and stromal (with stromal cells loosely referring to the non-haemopoietic cells of mesenchymal origin; Deans and Moseley 2000). The stromal system, as proposed by Owen (1985), was based on an analogy with the haematopoietic system, in which MSCs reside within the marrow, maintain a level of self-renewal, and give rise to cells that can differentiate into different connective tissue lineages as well as stromal tissues (Owen 1988).

Within the stromal environment, alkaline phosphatase positive (ALP*) reticular cells associate loosely with haematopoietic cells (Bianco 1993). These ALP* reticular cells
are thought to originate from cells that are destined to differentiate into osteoblasts but
are also capable of forming stroma. The presence of adipocytes in the postnatal
stroma depends on the stage of skeletal development, age, and the level of
haematopoiesis (Dexter 1982). It has been suggested that ALP+ reticular cells and
adipocytes are alternative phenotypes that are modulated by the marrow environment
(Bianco 1988).

The differentiation potential of the stromal cell system has also been widely studied
with respect to the mesenchymal connective tissues, in particular, bone tissues (Aubin
1998). Human stromal cells that had been depleted of circulating haematopoietic cells
by negative immunoselection with antibodies against monocytes/macrophages
(anti-CD14), endothelial cells (anti-CD31) and lymphocytes (anti-CD11a/LFA-1)
were shown to co-express genes characteristic of the osteoblastic lineage (alkaline
phosphatase, osteocalcin, and osteopontin) and adipocyte lineage (lipoprotein lipase),
indicating that stromal cells were uncommitted precursor cells (Rickard 1996).
Human marrow stromal fibroblasts are capable of forming colonies in vitro in the
presence of serum and these cells produce at least four growth factors:
platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF),
transforming growth factor β (TGF-β), and epidermal growth factor (EGF)
(Kuznetsov 1997a; van den Bos 1997). Pittenger et al. (1999) reported that approximately one-third of the initially adherent bone marrow-derived stromal colonies are pleuripotent and capable of differentiation into the osteogenic, chondrogenic and adipogenic lineages as demonstrated by lineage specific in vitro assays.

Kuznetsov et al. (1997b) demonstrated that in vivo transplants of all multicolonony-derived marrow stromal fibroblasts derived from multiple in vitro stromal cell colonies resulted in bone formation, whereas only 58.8% of fibroblasts formed bone. The same investigators recently presented evidence for a circulating osteogenic precursor in humans, although the circulating precursor demonstrates greater variability in clonogenic potential than precursors derived from bone and bone marrow. That is to say, the circulating precursors not only differentiate into bone cells, but also are more able to commit to other cell lineages (Kuznetsov 2001). The reported precursor frequency of osteoblast precursors in normal human bone marrow is approximately four per 100,000 nuclear cells (based on ALP$^+$ staining) and appears to correlate negatively with age and the existence of disease states such as osteoarthritis (Majors 1997).
Mesenchymal stem cell cultures are often defined as the non-haematopoietic adherent cell population which are obtained by direct plating of bone marrow. Marrow stroma may have relatively simple or complex cellular compositions depending on the growth media or plating substrate used (Deans and Moseley 2000). The majority of reported tissue culture conditions use relatively undefined media compositions containing foetal calf serum or other animal sera, this limits the supply of physiologic signals required for efficient attachment and differentiation as well as growth. However, ex vivo culture results in consistently reproducible stromal cell cultures that have been evaluated in human clinical studies for support of autologous haematopoietic engraftment (Lazarus 1995).

Standard conditions for generation of marrow-derived mesenchymal stem cell cultures have been reported (Bruder 1998b, Majumdar 1998). In brief, a bone marrow aspirate is collected and processed using density gradient centrifugation, from which light-density cells are taken and plated at a standard density in a Dulbecco's modified essential medium (DMEM) containing foetal calf serum. After allowing two days for adhesion to non-coated polystyrene, non-adherent cells are removed, and a feeding schedule established for a 14-day primary expansion period of adherent colonies. At this time, near-confluent cultures can be processed further by trypsinisation and
expansion through sequential passages. Cells may be expanded by 40 generations and still retain their multipotent mesenchymal lineage capability, although growth rates become reduced. The expanded mesenchymal stem cells exhibit a finite lifetime and do not display properties of immortalised cells (Deans and Moseley 2000).

In terms of mobilisation of circulating mesenchymal stem cells, investigators have tried to detect circulating cells exhibiting the multilineage mesenchymal stem cell phenotype. It has been described by Chesney et al. (1997) that a fibroblast-like cell, termed fibrocyte, is found in the circulating blood. These fibrocytes are reported to be CD34⁺, CD45⁺, CD13⁺, and to be capable of synthesizing collagen. In addition, these cells express class II HLA molecules, the co-stimulatory molecules CD80 and CD86, and the adhesion molecules CD11a, CD54 and CD58. These cells are also demonstrated to induce T-cell responses consistent with a dendritic cell function. Previous studies from this group have shown that this circulating cell can be recruited where it aids the tissue repair processes (Bucala 1994). Additional evidence for circulating mesenchymal stem cell populations is provided by Ferrari et al. (1998), who reported that the transfer of genetically marked (β-galatosidase) bone marrow could lead to genetically marked muscle cell progenitors in mice. These precursors respond to signals for muscle regeneration with proliferation and differentiation into
mature myocytes, indicating that the mesenchymal muscle progenitor is derived from a bone marrow-derived precursor or stem cell.

In terms of the characteristics of human mesenchymal stem cells, hMSC cultures contain a homogenous population of fibroblast like cells which have a population doubling time of 33 hours and exhibit a large but variable ex vivo expansive potential (Conget and Minguell 1999). It has been reported that while some MSC preparations can be expanded by over 15 cell doublings, others cease replicating after about 4 cell doublings (Bruder 1997a, Digirolamo 1999, Phinney 1999). In addition, some investigators reported that human mesenchymal stem cells could maintain the potential to differentiate into osteogenic lineage even after 40 doublings in culture. Cryo-preserved MSCs are also able to differentiate into osteogenic lineage after being thawed (Bruder 1997a, Jaiswal 1997). hMSCs that are highly expanded (>40 doublings) do loose their multipotentiality and approach senescence and at this stage numerous apoptotic features are present in culture (Conget and Minguell 1999, Digirolamo 1999).

Cell cycle studies on isolated human mesenchymal stem cell culture have revealed the presence of a fraction (20%) of cells with a quantitative pattern of RNA and DNA
typical of quiescent (G0) cells. These cells can be isolated by a negative selection procedure using 5-fluorouracil, which selects a population of more than 90% G0 cells, expressing the gene for ornithine decarboxylase antizyme (Minguell 2000). The resting condition, together with a selective immunophenotype and the absence of commitment markers, gives experimental support to the hypothesis that a “rare” mesenchymal stem cell in the bone marrow is capable of self-renewal and differentiation into various mesenchymal lineages.

The antigen profile of MSCs is not unique, and antigens are common to mesenchymal, endothelial, epithelial and muscle cells (Haynesworth 1992a; Galmiche 1993; Conget and Minguell 1999; Pittenger 1999). Moreover, since hMSCs do not express typical haematopoietic lineage markers such as CD14, CD34 and CD45 (Conget and Minguell 1999; Pittenger 1999), it supports the concept that the bone marrow hosts at least two main different stem/progenitor cells which can give rise to mature haematopoietic and mesenchymal cells (Prockop 1997 and Gerson 1999).

The extended cytokine expression profile of hMSCs, which includes several haematopoietic and non-haematopoietic growth factors, interleukins and chemokines, suggests that hMSCs contribute to the marrow microenvironment by providing
inductive and regulatory signals necessary for the development of haematopoietic
cells as well as for stromal cells, including hMSCs themselves (Haynesworth 1996;
Majumdar 1998). It has been postulated that these hMSCs are under the control of
autocrine or juxtacrine loops because of the expression of the numerous growth factor
and cytokine receptors on them (Pittenger 1999). Additional evidence for the dynamic
function performed by hMSCs in the marrow microenvironment is given by data
revealing their capability to produce and organise an array of extracellular matrix
molecules (Chichester 1993). Moreover, hMSCs express several receptors that are
associated with matrix- and cell-to-cell adhesive interactions (Conget and Minguell
1999; Pittenger 1999), which enable the attachment, adhesion and homing of MSCs at
the desired locations.

As described above, the in vitro potential of MSCs to differentiate into osteoblasts,
chondrocytes, myotubes and haematopoietic-supporting stroma, has been
demonstrated (Caplan 1994; Wakitani 1995; Jaiswal 1997; Dennis 1999). However,
the cellular and molecular events associated with differentiation pathways are not
clearly understood. It seems that commitment to the osteo-chondrogenic lineage
requires the gene expression of Cbfa-1 (Ducy 1997), while commitment into
adipogenic lineage requires the expression of PPAR γ 2 (Tontonoz 1994). Also, there
is evidence that hMSCs commit to osteogenic or adipogenic lineage after being exposed to osteogenic supplements and thus is regulated by mitogen-activated protein kinase (Jaiswal 2000). Subsequent maturation along these pathways includes the expression of alkaline phosphatase, osteopontin, osteocalcin and collagen I in the osteocytic lineage; collagen II and collagen IX in the chondrocytic lineage; aP2, leptin and lipoprotein lipase in the adipocytic lineage (Gori 1999; Pittenger 1999). Analysis at the gene expression level using RT-PCR has shown that hMSCs differentiate in vitro, according to the stimuli applied, into the desired lineage that are committed and not capable of de-differentiation, but not into cells expressing multiple lineages (Pittenger 1999).

Although diagrams for a hierarchy of mesenchymal progenitors from a putative mesenchymal stem cell have been published (Bruder 1994; Caplan and Dennis 1996; Bruder 1997b, Bordignon 1999), information explaining how gene and gene transcription are controlled and how this leads to cell differentiation from multipotent mesenchymal progenitor cells are lacking. More works need to be done to fill in the gaps on these issues (Minguell 2000). A summary of differentiation of human MSCs is shown in Figure 1-2.
Figure 1-2. A diagram showing differentiation of human mesenchymal stem cells.

Recent data have shown that, in addition to adult human bone marrow, umbilical cord blood is also a source of MSCs (Erices 2000). These cells exhibit an
immunophenotype, a population of quiescent cells and a differentiation potential similar to that of bone marrow-derived MSCs. The content of hMSCs is higher in preterm than in term cord blood, and thus has also been observed for haematopoietic progenitors (Shields and Andrews 1998), which suggests that haematopoietic and mesenchymal progenitors travel early during development, probably from foetal haematopoietic sites to the newly formed bone marrow via cord blood (Tavassoli 1991).

Given the promising features of adult stem cells for the development of new cell therapies (Weissman 2000), researchers have pursued numerous investigations to which have stimulated therapeutic utilisation of MSCs. The first clinical trials have revealed that systemic infusion of ex vivo expanded MSCs is feasible and safe in cancer patients in the short term (Lazarus 1995; Koc 2000). Also, the safety and influence of systemic infusion of MSCs on whole body physiology has been explored (Haynesworth 1998). However, there is yet no conclusive evidence to support the contention that transplanted MSCs may have a positive impact on the management of lymphohaematopoietic or cancer patients (Koc 2000). More positively, increased growth rate and reduced frequency of bone fractures were also observed in children with osteogenesis imperfecta when MSCs were given intravenously (Gerson 1999).
These changes, detected three months after marrow transplantation, were associated with the engraftment of functional MSCs from the systemically transplanted marrow (Gerson 1999). In summary, the last five years have been the scene of a substantial improvement in our understanding of the biology and the potential clinical utilisation of hMSCs, in particular, with the tissue engineering approach to repair the musculoskeletal tissues.

1.4.3 SCAFFOLDS FOR BONE TISSUE ENGINEERING

Scaffolds can provide necessary support for cells to maintain their differentiated function and the shape of the scaffold can define the ultimate morphology of new bones formed by tissue engineering techniques (Crane 1995). Before considering the desired features of potential tissue engineering materials, it is useful to understand two concepts of bone regeneration for tissue engineering constructs, specifically osteoinduction and osteoconduction (Burg 2000). Osteoinduction is defined as the ability to cause pluripotent cells, from a non-osseous environment to differentiate into chondrocytes and osteoblasts, culminating in bone formation (Urist 1967). An osteoinductive material guides repair in a location that would normally not heal if untreated (Bostrom and Mikos 1997). Osteoconduction supports ingrowth of capillaries and cells from the host into a three-dimensional structure to form bone.
osteoconductive material guides repair in a location where normal healing will occur if untreated.

Taking the concept of osteoconduction and osteoinduction into consideration, the desirable qualities of a bone tissue-engineering scaffold design have been postulated by several researchers (Levine 1997; Brekke and Toth 1998; Peter 1998). First of all, the scaffold must be able to promote maximal bone ingrowth through osteoinduction and/or osteoconduction. This scaffold must not induce soft tissue growth at bone/scaffold interface. In addition, it should 1) not exert any detrimental effects on surrounding tissues; 2) be sterilisable without loss of properties; 3) be absorbable with biocompatible components; preferably it should be absorbed in a predictable manner inversely to the amount of bone formation; 4) be malleable and adaptable to irregular bone defect sites. At the early stages after implantation the scaffold should possess proper mechanical and physical properties. Most important of all, it should be easy to apply and available to surgeons at short notice.

There are several categories of biomaterials that have been extensively studied which are used as scaffolds for bone tissue engineering. These include naturally derived polymers, synthetic polymers and ceramics (Hollinger and Battistone 1986,
Ripamonti and Tasker 2000). Demineralised bone matrix is a typical example of naturally derived polymers. In the early 1960s, Urist and several co-workers realised that demineralised bone consistently induced bone formation in ectopic tissues of experimental animals (Van de Putte and Urist 1965; Van de Putte and Urist 1966). A hydrochloric acid extraction process decalcified the bone matrix (Urist 1967; Urist 1979). This decalcified matrix was shown to possess inherent osteoconductive and osteoinductive properties (Urist 1967). It was subsequently postulated that cortical bone is the preferred choice for demineralised bone matrix (DBM) synthesis as it is more osteoconductive with a lower antigenic potential than cancellous bone (Urist and Dawson 1980). The histological process by which new bone is formed under the influence of DBM in ectopic sites has been described (Urist 1967). The process imitates endochondral ossification (Harakas 1984). It has also been pointed out that the way DBM is processed can greatly influence the final osteoinductive ability (Russell and Block 1999).

There has been a persistent orthopaedic interest in DBM because of its therapeutic potential in the treatment of bony defects, non-unions and its application in arthrodesis procedures. In animal experiment, DBM can achieve union in extensive bone defects (Gepstein 1987). In that study, defects of more than 50% of the length of
the femur were created on rats, and, after 35 days, 71% of the animals in which the gaps were packed with DBM, achieved bone union. In a review of the clinical results in 21 studies using DBM as a substitute for autologous bone graft in various orthopaedic procedures (Russell and Block 1999), it was reported that over 80% of the studies demonstrated favourable results. These results were obtained from a number of different studies and where different methods were used to sterilise the DBM. There are, however, problems using DBM as a scaffold for bone tissue engineering applications. There is no initial mechanical strength that can be rendered by DBM, therefore its use for scaffold of weight-bearing long bones is limited. In addition, DBM cannot guide three-dimensional development of the tissue structure. The sterilisation procedure can also alter the osteoinductive ability of DBM. For example, sterilisation of DBM with ethylene can result in complete loss of its osteoinductivity, although the osteoconductive ability would remain (Gogolewski and Mainil-Varlet 1997).

The other naturally derived polymers which can be used as scaffolds of tissue-engineered bones is collagen. As collagen possesses no inherent structural mechanical properties, engineering modification is needed. It has been reported that porous collagen foams can be treated with calcium solution to allow the deposition of
calcium phosphate and improve its mechanical integrity (Yaylaoglu 1999). This technique shows promise in chondrocyte culture and has great potential for bone application as well. However, it is not known whether this system has long-term stability in culture. It has also been reported that collagen sheets can be used as the basis for composite bone tissue-engineering scaffolds (Du 1999). In that study, hydroxyapatite was precipitated onto the surfaces of commercially available collagen sheets. Bone fragments were then placed along the surfaces, and the sheets were rolled into tubes. Cell migration was demonstrated from the bone fragment into the matrix, suggesting that the material is bioactive. Again, the properties of this material are not sufficient to support the initial mechanical stability.

Another category of biomaterials that can be used for tissue-engineered bone scaffolds are the synthetic polymers. Synthetic injectable materials are finding appeal as bone tissue-engineered scaffolds, due in part to their minimally invasive implantation. It has been reported that photopolymerisable materials can be injected as liquids and photopolymerised to localize the material (Elisseeff 1999). Although it was tested for transdermal application in that study, with the increased interest in minimally invasive procedures, this may have orthopaedic application as well.
Polyglycolide (PG) fibrous, non-woven mesh is another tissue-engineering candidate. Clinically well known and having the advantage of fast absorption, this material has been applied to almost every area of tissue engineering, including bone regeneration (Puelacher 1996). PG mesh demonstrates relatively low mechanical integrity in vitro, therefore it would be inappropriate to use it alone as a bone tissue-engineering construct (Holder 1997). By combining PG with a second reinforcing material, a stable construct can be formed. This has been accomplished in the past using a polylactide (PL) solution to bond the mesh (Mooney 1996). More recently, a specific bone application has been accomplished by coating a PG-based tube with PL (Puelacher 1996). In that study, osteoprogenitor cells were seeded on PG mesh which in turn, was applied to the hollow portion of the stabilised tubes. These constructs show promise as long-bone defect replacements in a rat femur defect model. New synthetic polymers are also of great interest as potential cellular scaffolds. It has been reported that poly-anhydride-co-amides are suitable candidates for bone tissue engineering scaffolds (Attawia 1999). These materials are absorbed by surface erosion, thus having the advantage of a more predictable mass loss. Furthermore, they have high mechanical strength and rigidity, it is reported that their compressive moduli range from 10 to 60 MPa (Attawia 1999).
The most extensively studied biomaterials for scaffolds of bone tissue engineering are ceramics. The absorbable inorganic materials that have been investigated include CaCO$_3$ (argonite), CaSO$_4$·2H$_2$O (plaster of Paris), and Ca$_3$(PO$_4$)$_2$ (beta-whitlockite, a form of TCP) (Hollinger and Battistone 1986). Among these ceramics, the most widely studied calcium phosphate ceramics are TCP, hydroxyapatite (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$), and the newest tetracalcium phosphate (Friedman 1998). The appeal of the calcium phosphates rests largely with their biocompatibility. Since they are protein free, minimal immunologic reactions, foreign body reactions, or systemic toxicity have been reported with their use (Hammerle 1997). It has been shown that hydroxyapatite ceramic has the highest biocompatibility in human bone marrow cell cultures in comparison with pure titanium and ultra-high molecular-weight polyethylene, two other commonly used orthopaedic biomaterials (Wilke 1998).

The advantage of these materials is probably related to their chemical composition, which, when they are implanted in bone, allow calcium and phosphate ions to be derived from the scaffold and/or surrounding bone. The compositions of any solids deposited on the surface of the calcium phosphate scaffold are believed to be determined by the surrounding medium, and ultimately, in bone, this medium would generate calcium phosphate solids in the form of biologic apatite (Manley 1993).
Because of the above mentioned characteristics, clinically hydroxyapatite has been used as a coating on the femur stem of hip prostheses (Oonishi 1991) and bone graft substitutes (Holmes 1986; Holmes and Hagler 1988; Johnson 1996). Numerous in vitro and animal studies about the feasibility of using hydroxyapatite as scaffold materials for osteogenic cells have also been carried out. It has been proved that bone marrow cells, when loaded into hydroxyapatite blocks, can induce heterotopic osteogenesis and repair bone defects in rats (Maniatopulos 1988; Ohgushi 1989a; Ohgushi 1989b; Ohgushi and Okumura 1990; Goshima 1991a; Goshima 1991b; Ohgushi 1992; Kadiyala 1997) and in other animals such as canine (Bruder 1998b) and sheep (Kon 2000). The pre-cultured cell-hydroxyapatite complex (prefabricated osteogenic hydroxyapatite) has been shown to possess immediate bone forming capacity in vitro (Yoshiwaka 1996). The osteogenic potential of human bone marrow cells cultured on hydroxyapatite has also been demonstrated (Torquet 1999). The advantages of porous hydroxyapatite are that it provides an ideal osteoconductive surface for three-dimensional cellular development, which is essential for ex vivo formation of human bone (Kale and Long 2000). In addition, it has been shown that hydroxyapatite induces the osteoblastic phenotype expression of bone marrow-derived stem cells (Okumura 1997). Furthermore, it is possible to coat the hydroxyapatite with other biomaterials, for example, fibronectin, to enhance the cell
adhesion onto the hydroxyapatite surface (Dennis 1992).

Lastly, one potential candidate material which may be used as scaffolds of tissue engineered bone tissues is fibrin glue. Fibrin glue is a physiologically relevant matrix whose principal component, fibrin, has a fundamental role in the process of blood clotting and wound healing. Fibrin glue, a composite of fibrinogen and thrombin, is a potentially suitable biological vehicle for cell transplantation because it has proven biocompatibility (Ciano 1986), biodegradability (Lambrecht and Klinger 1990) and binding capacity to cells (Keller 1985). Fibrin-stabilizing factor XIII contained in fibrin glue favors migration of undifferentiated mesenchymal cells on the highly cross-linked structure of the glue, and this factor has been shown to enhance the proliferation of these cells (Kasai 1983). The rate of resorption of fibrin glue can be controlled by varying the concentration of the fibrinolytic inhibitor, apoptin (Meinhart 1999). This keeps the cells in place, increases cell survival, and improves the immediate mechanical properties of the implant. The fibrin extracellular matrix remains in situ while the cells proliferate and differentiate into new tissue, before the scaffold is completely resorbed (Horch 1998 and Llull 1999). Most important of all, the fibrin glue promotes angiogenesis via chemotactic and mitogenic stimuli that promotes cell migration, proliferation and matrix synthesis (McGrath 1990). Recently
it has been demonstrated that fibrin glue can be used as scaffolds for periosteal cells or calvarial-derived osteoprogenitor cells to repair bone defects in animal experiments (Tholpady 1999; Isogai 2000; Perka 2000). Finally, hydroxyapatite granules can be mixed with fibrin glue to be used as bone graft substitutes and it has been shown that hydroxyapatite granules mixing with fibrin glue had higher bone regeneration rate than using hydroxyapatite granules alone (Oberg and Kahnberg 1993).

1.4.4 GROWTH FACTORS

Besides cells and scaffolds, signalling molecules also play a vital role in tissue engineering of bone tissues. Bone morphogenetic proteins (BMPs) play a crucial role in growth and differentiation of bone cells (Kingsley 1992; Reddi 1992; Reddi 1994). Urist made the key discovery that demineralised bone induced bone formation when implanted subcutaneously or intramuscularly in animals (Urist 1965; Urist 1976). Bone induction is, in fact, a sequential multi-step cascade (Reddi and Huggins 1972; Reddi 1981). The key steps in this cascade are chemotaxis, mitosis and differentiation (Reddi 1998). Chemotaxis is the directed migration of cells in response to a chemical gradient of signals released from the insoluble demineralized bone matrix (Reddi 2000). The demineralised bone matrix is predominantly composed of type 1 insoluble collagen and it bonds plasma fibronectin (Weiss and Reddi 1980). Fibronectin has
domains for binding to collagen, fibrin, and heparin. When DBM is implanted into the
thigh muscles of the rats, responding mesenchymal cells attached to the collagenous
matrix and proliferated on day 3 (Reddi and Anderson 1976). Chondroblastic
differentiation was evident on day 5, chondrocytes on day 7-8, and cartilage
hypertrophy on day 9. There was concomitant vascular invasion on day 9 with
osteoblast differentiation. On days 10-12, alkaline phosphatase was maximal.
Osteocalcin, bone $\gamma$-carboxyglutamic acid containing gla protein (BGP), increased
on day 28. Haematopoietic marrow differentiated in the ossicle and was maximal by
day 21. This entire sequential bone development cascade is reminiscent of cartilage
and bone morphogenesis in the developing limb bud (Reddi 1981). Hence, it has
immense implications for isolation of inductive signals initiating cartilage and bone
morphogenesis (Reddi 1998). In fact, a systematic investigation of the chemical
components responsible for bone induction was undertaken and inductive signals
were identified and isolated successfully (Reddi 2000). Subsequent purification
studies of these bone-inductive proteins from bovine bone resulted in the
identification of many members of BMPs (Wozney 1988; Ozkaynak 1990).
Complementary DNA (cDNA) cloning of BMPs revealed that BMPs form a unique
subfamily within the transforming growth factor-$\beta$ (TGF-$\beta$) superfamily (Sakou
1998). Members of the BMP family play a crucial role in regulating the growth,
differentiation and apoptosis of various cell types including osteoblasts, chondroblasts, neural cells and epithelial cells (Reddi 1992; Kingsley 1994). There is growing evidence to suggest that BMPs and their receptors play an essential role in the formation of skeletal tissues and during bone regeneration following fracture (Schmitt 1999).

Members of TGF-β superfamily, including BMPs, encode secreted polypeptides that share common structural features (Kingsley 1994). Up to now, more than forty of the BMP proteins have been isolated (Reddi 1997), including BMP-2 to BMP-6 (Wozney 1988; Celeste 1990), osteogenic protein-1 (OP-1) and OP-2 (also termed BMP-7 and BMP-8, respectively) (Ozkaynak 1990; Ozkaynak 1992), and growth/differentiation factor-5 (GDF-5) to GDF-7 (BMP-14 to BMP-12, respectively) (Storm 1994). GDF-5 and GDF-6 are also called cartilage-derived morphogenetic protein (CDMP)-1 and -2, respectively (Chang 1994).

BMPs bind and initiate a cell signal through a transmembrane receptor complex formed by types I and II serine/threonine kinase receptor proteins (Schmitt 1999). Type-I (BMPR-IA or BMPR-IB) and type-II (BMPR-II) receptor proteins are distinguished on the basis of their molecular weights, the presence of a glycine/serine
CHAPTER 1

rich domain located on the type-I receptor, and the ability to bind a particular ligand. Individual receptors have low affinity for BMPs; however, as a heterotetrameric complex, high-affinity bonding is achieved (Liu 1995; Nohno 1995; Rosenzweig 1995). Evidence suggests that the type-II receptors are active continuously (autophosphorylating) and function upstream of the type-I receptors but cannot independently initiate cell signals (Wrana 1994). On binding BMPs 2, 4 and 7 (OP-1), the type-II receptor kinase transphosphorylates the type-I receptor at the glycine/serine rich region; this event generates an intracellular response (Massague 1996). Specificity in signaling appears to be determined primarily by the type-I receptor (Carcamo 1994).

Native BMP is present in cortical bone in minute amounts, approximately 1 to 2 \( \mu g \) per kilogram cortical bone (Luyten 1989; Sampath 1990; Wang 1988). Its extraction is difficult and not entirely predictable, and it always contains minute contaminants (Riley 1996). Successful cloning of BMPs 1, 2, 3 and 4 was first reported in 1988 (Wozney 1988) and other BMPs continued to be cloned thereafter (Celeste 1990; Luyten 1989; Ozkaynak 1990; Sampath 1990; Wang 1988; Woznwy 1989). The application of recombinant DNA technology to the BMPs, with use of a Chinese hamster ovary cell line, will allow for a supply of almost unlimited amounts of
recombinant human BMP (rhBMP) for further study and clinical applications (Israel 1992).

Recombinant human BMP-2 is a 32-kd homodimeric glycoprotein, though heterodimer seems to exist in native BMP. Each monomer in the active form consists of 114 amino acids (Riley 1996). In amino acid sequence, BMP-2 displays 74% similarity to the dpp protein in the *Drosophila* (Padgett 1987). This similarity in the developmental signalling peptide to the distant species suggests that BMP-2 plays a regulatory role in embryogenesis (Padgett 1993; Sampath 1993). Detailed in situ hybridization analysis and immunohistologic studies (monoclonal antibodies specific to BMP-2) have shown that BMP-2 is present during embryonic development in a spatial and temporal pattern consistent with involvement in skeletogenesis (Schildauer 1994). More evidence for the role of BMP-2 in extraskeletal organogenesis comes from in situ hybridisation studies in the mouse (Lyons 1990). BMP-2 RNA is expressed in a variety of embryonic epithelial and mesenchymal tissues separate from the skeletal system (Lyons 1995a). The localisation of transcripts of high affinity BMP receptors to developing skeletal and neural tissues strongly supports a role of BMPs in aspects of morphogenesis (Lyons 1995b).
BMP-2 is present during fracture repair and has specific cellular localisations (Bostrom 1995; Barnes 1999). Human bone morphogenetic protein-2 is involved in the differentiation of mesenchymal stem cells (Hanada 1997; Ju 2000). Numerous studies have been done to prove the in vitro and in vivo safety and efficacy of rhBMP-2 (Boden 1999). Extensive data show that rhBMP-2 is a morphogen, not a mitogen, and will induce cells to differentiate and form endochondral bone in ectopic and heterotopic locations (Aspenberg 1996; Aspenberg and Turek 1996; Wang 1990; Yamaguchi 1991). The original preclinical studies largely tested repair of critical sized defects in rats, dogs, rabbits and sheep (Gerhart 1993; Itoh 1998a; Itoh 1998b; Lee 1994; Kirker-Head 1995; Kirker-Head 1998; Mori 2000; Yasko 1992; Zegula 1997; Zellin and Linde 1997a; Zellin and Linde 1997b). Later, evidence became available describing the efficacy of rhBMP-2 for posterolateral spinal fusion in rabbits, dogs, and most recently, non-human primates (Boden 1996; Fischgrund 1997; Hollinger 1996b; Martin 1999; Muschler 1994; Sandhu 1995; Sandhu 1996; Sandhu 1997; Schimandle 1995). In addition, rhBMP-2 delivered in an absorbable collagen sponge has been successfully used as a graft substitute inside Titanium interbody fusion devices in non-human primates (Boden 2000; Sandhu 2000). Recently, rhBMP-2 has also been reported to be effective in enhancing dental implant fixation, alveolar bone augmentation (Wikesjo 2001) and treating oromaxillofacial osseous defects (Boyne
The other BMP with extensive in vitro and in vivo data is rhBMP-7, otherwise known as osteogenic protein-1 (OP-1). The human OP-1 cDNA was cloned by means of oligonucleotide probes based on the bovine OP peptide sequences and their relationship with Drosophila Dpp and Xenopus Vg-1 (Cook and Rueger 1996). The recombinantly expressed protein is secreted as a mature homodimer that has been cleaved proteolytically between arginine 292 and serine 293, which is immediately preceded by an Arg-X-X-Arg proteolytic processing signal (Jones 1994). This signal sequence seems to be a common processing site for all family members. The resulting mature homodimer (32-36 kd) is composed of two subunits, each of which contains 139 amino acids (Sampath 1992). Although there are four potential N-glycosylation sites in the OP-1 molecule (three in the mature domain and one in the predomain), only one site in the mature domain in addition to the site in prodomain is glycosylated.

Comparison of the amino acid sequences of the TGF-β superfamily within the highly conserved seven cystein domain indicated that BMP-7 is a member of a subfamily different than BMP-2 and is more closely related to BMP-5 and BMP-6 in
amino acid structure (Ozkaynak 1990). OP-1 is most closely related to the BMP-5/6 gene products (88%, 87%), to a lesser extent BMP-2/4 (60%, 58%), and to a much lesser extent BMP-3 (42%) and the TGF-βs themselves (38%-35%) (Griffith 1996). Although Drosophila does not contain bone, it has been shown that purified recombinantly produced mature disulfide-linked homodimers of Dpp and 60A can induce the formation of new bone in the rat subcutaneous bone induction assay (Sampath 1993).

Implantation of purified recombinant human OP-1 with a collagen carrier in subcutaneous sites in rats induces a sequence of cellular events that lead to the formation of fully functional new bone (Sampath 1992). In animal studies, OP-1 has been successful in various preclinical models including segmental defect repair in dogs and nonhuman primates, posterolateral spinal function in dogs and other animal models (Asahina 1993; Cook 1994a; Cook 1994b; Sampath 1992). Recently, the results of a clinical trial have shown that BMP-7 is as effective as autologous bone graft in treating tibial non-union (Friedlaender 2001). In this study, rhOP-1 was delivered to the non-union site with bovine bone-derived type 1 collagen. The authors found that in the control group, more than 20% patients treated with autografts had chronic donor site pain following the procedure. Therefore, the advantage of using
Another growth factor which is important in tissue engineering of bone is fibroblast growth factor (FGF), in particular, FGF-2. FGF-2 is also known as basic fibroblast growth factor (bFGF). It has been reported that FGF-2 accelerates fracture healing in various animal experiments (Nakamura 1996, Nakamura 1998a, Radomsky 1999, Kawaguchi 2001). The details are reviewed in Chapter 2. It has been reported that a single local injection of FGF-2 at the fracture site enhanced fracture union (Kato 1998) and accelerated distraction osteogenesis (Okazaki 1999). However, the effects of this short-term exposure of FGF-2 on MSCs are not clear. Therefore, it is valuable to investigate the effects of short exposure of FGF-2 on MSCs.
1.5 STUDY DESIGN

Since tissue engineering of bone regeneration is such a rapid developing discipline, it would be extremely useful if the principles of tissue engineering in bone regeneration can be applied to individuals receiving chemotherapy to overcome the possible detrimental effects chemotherapy will cause. An in vivo animal experiment will be performed to test the hypothesis that use of mesenchymal stem cell-based tissue engineering approach facilitates bone regeneration.

In order to establish a tissue engineering approach to enhance bone regeneration in patients with chemotherapy, a series of experiments are designed and the hypotheses of this thesis are to be tested in the following chapters. In Chapter 2, the effects of short exposure of FGF-2 on MSCs are to be investigated. The reason why FGF-2 is chosen is that recombinant human BMP-2 (rhBMP-2) and OP-1 (rhOP-1) are patented by Genetics Institute and Stryker Biotech, respectively. Both companies are in the United States. So far neither rhBMP-2 nor rhOP-1 is licensed in the United Kingdom and we are not able to get hold of either of the growth factor for research. On the contrary, FGF-2 is commercially available. Quantitative analysis of the detrimental effects of chemotherapy on bone cells and skeletal tissues is important and the effects
of chemotherapy agents on MSCs and the skeleton are to be explored in Chapter 3 and 4, respectively. To what extent chemotherapy will affect bone regeneration is also an important question and in Chapter 5, the detrimental effects of chemotherapy on fracture healing are to be investigated. Finally, in order to understand whether MSC-based tissue engineering approach will enhance bone regeneration, the effects of applying MSCs with fibrin glue at the bone defect site will be investigated in Chapter 6.
Chapter Two

EFFECTS OF SHORT EXPOSURE OF FIBROBLAST GROWTH FACTOR-2 ON MESENCHYMAL STEM CELLS
2.1 Introduction

2.2 Materials and methods

2.3 Results

2.4 Discussion
CHAPTER 2

2.1 INTRODUCTION

Cells, scaffolds and signalling molecules play an important role in tissue engineering. In terms of tissue-engineered bones, bone morphogenetic proteins such as BMP-2 and BMP-7 (OP-1) have been extensively studied. However, another growth factor, fibroblast growth factor-2 (FGF-2), also called basic fibroblast growth factor, has been shown to influence proliferation and differentiation of mesenchymal stem cells. It has been reported that a 6-day exposure of FGF-2 to dexamethasone-treated rat mesenchymal stem cells stimulated cell growth and induced osteoblastic differentiation; indicating that FGF-2 enhances both mitogenic activity and osteogenic development (Hanada 1997; Scutt and Bertram 1999).

In human MSCs, it has also been demonstrated that messenger RNA of FGF receptors were expressed and these receptors were detected on the surfaces of these cells. In addition, it has been shown in vivo that FGF-2 increased the rate of proliferation in human MSCs (van den Boss 1997) whereas, disruption of FGF-2 gene resulted in decreased bone mass and decreased bone formation in adult mice (Montero 2000). Stimulation of endosteal bone formation by local intraosseous application of FGF-2 has been shown in rats (Nakamura 1997a). Recombinant human fibroblast growth factor-2 has been shown to enhance fracture healing in rats (Kawaguchi 1994) and
rabbits (Nakamura 1997b), dogs (Nakamura 1998a) and nonhuman primates (Radomsky 1999). Local application of FGF-2 incorporated into a minipellet was also shown to increase the rate of fracture healing in segmental bone defects in rabbits (Inui 1998).

Importantly, it has been reported that a single local injection of recombinant fibroblast growth factor into a bone defect can stimulate healing of segmental bone defects (Kato 1998) and stimulate bone formation in callotasis bone lengthening (Okazaki 1999) in rabbits. Kato et al. (1998) showed that 50, 100, 200 and 400 μg of recombinant human FGF-2 in 0.1 ml of saline solution injected into a 3 mm defect have had a dose-dependent stimulatory effect increasing bone volume and bone mineral content. This finding is important because of the simplicity and the cost-effectiveness of the procedure. However, the mechanism by which a single local injection of FGF-2 stimulates fracture healing or facilitates bone consolidation in callotasis is still unclear. According to the unpublished observation of Okazaki et al. (1999), only less than 10% of FGF-2 in saline solution remains at the injection site 24 hours after injection and they postulate that it could be either the stimulation of mesenchymal cell proliferation or recruitment of those cells from less differentiated progenitors. Very little in the literature can be found about the effects of short
exposure of FGF-2 on mesenchymal stem cells. My study was therefore designed to elucidate the effects of short exposure of FGF-2 on the proliferation and osteogenic differentiation of mesenchymal stem cells. We hypothesize that there is dose- and exposure-time dependent effect of FGF-2 on the proliferation and the osteogenic differentiation of mesenchymal stem cells.
2.2 MATERIALS AND METHODS

Cells, C3H10T1/2 from a pluripotent murine embryonic stem cell line, was purchased from American Type Cell Collection (Vanassas, VA, USA, ATCC No 226) and was used for this study. The reason that cells from a commercially available cell line were used instead of primary animal or human MSC was to avoid the variability of the primary cells confounding the experimental results. FGF-2 was purchased from Sigma-Aldrich (Dorset, UK). Unless otherwise specified, the chemicals and media used in this study were purchased from Sigma-Aldrich (Dorset, UK).

The study consisted of two parts. In the first part, the effects of different concentrations of FGF-2 on MSCs were investigated. One hundred thousand cells were seeded into each well of six well plates. The culture media used was composed of Dulbecco's Modified Eagle Medium (DMEM) plus 10% foetal calf serum and 1% penicillin/streptomycin. After 12 hours, the cells attached to the plastic surface, FGF-2 was added into the wells at the concentration of 0.1, 1, 10 and 100 ng/ml. The media for the control group contained no FGF-2. After 24 hours, media was changed and fresh media containing no FGF-2 was added into all groups. Media was then changed twice a week and the cells were kept for three weeks. Evaluations of proliferation by $^3$H-Thymidine incorporation assay were done on days 2, 7, 14, and 19. In each group
there were six replicas at each time period. Briefly, 1 µCi/ml titrated thymidine (3H-TdR, Amersham Life Sciences, UK) was added to the culture system 24 hours before analysis. Then the cells were collected and freeze-thawed. One hundred µl of freeze-thawed sample was added to each well of a 96 well Millipore filter plate in which the wells were rinsed beforehand with 200 µl of trichloroacetic acid (TCA) at 4°C. After the cell lysates were added, 100 µl of 20% TCA was also added to the wells and the plate was incubated in 37°C for 30 minutes. After 30 minutes of incubation, TCA was aspirated, the wells were rinsed with 10% TCA twice. The wells were left to dry. The precipitates were then collected from the 96 well plates by punching the filter paper into scintillation vials. Five hundred µl of 0.01 M KOH was added to the scintillation vial and the vials were placed on a shaking mixer for 2 hours. Scintillant was then added and the radioactivity emitted by the radio-labeled thymidine, which indicated the amount of newly formed DNA, was measured. The results were normalised by total DNA and expressed as counts per minute per µg of DNA. The detailed procedure sheet is in Appendix I.

Evaluations of osteogenic differentiation by Alkaline phosphatase assay were also performed on day 2, 7, 14 and 19, using the method described by Oreffo et al. (1998). Alkaline phosphatase activity was determined using a colorimetric assay at the
wavelength of 405 nm to measure hydrolysis of p-nitrophenol phosphate (Beresford 1992) in a spectrophotometer. Briefly, cell lysates and controls were removed from the freezer on the day before the assay was to be performed and left to thaw at 4°C overnight to allow the complete regeneration of enzyme activity. Then 50 μl of cell lysates was added to the sample cups used in the spectrometer (Cobas Bio, Roche Diagnostica, Germany). Then 0.25 ml of working reagent, consisting of 8mls of diethanlamine buffer and one bottle of p-nitrophenol phosphate powder (Randox), was added to each sample cup. Total DNA was measured using Hoescht 33258 dye reagent (Rago 1990). Alkaline phosphatase was expressed as nmol p-nitrophenol/hour/μg DNA.

Osteocalcin assay was carried out on cells which were in culture for 21 days using a radioimmunoassay (Power and Fottrrell 1991). The reason why osteocalcin test was only performed on Day 21 was that osteocalcin is a late osteogenic marker and it is not expressed at the early stages of osteogenic differentiation (Lian 1998). Osteocalcin test kit (Ocatest, Brahms Diagnostica, Berlin, Germany) was used. ¹²⁵I-labeled osteocalcin was offered in the kit and competed with osteocalcin in the samples for antibody against osteocalcin.

Type 2 collagen assay was qualitatively performed on day 11. Monoclonal antibody of
type 2 collagen was obtained from Development Studies Hybridoma Bank in the University of Iowa, USA. After the media were removed from the wells, they were washed with PBS three times. Then the samples were fixed with 4% formaldehyde/PBS at 37°C for 15 minutes. The fixative was removed and a further wash with PBS followed. Permeabilizing buffer was added and then the samples were incubated for 5 minutes at 4°C. The permeabilizing buffer was removed and replaced with 1% bovine serum albumin (BSA)/PBS and was left in 37°C for 5 minutes. The 1% BSA/PBS was removed and primary antibodies (made up in 1% BSA/PBS) was added and left for 1 hour. The unbound primary antibodies were then removed using 1% BSA/TWEEN. A secondary antibody, rabbit IgG, made up in 1% BSA/PBS, was added and left at 37°C for one hour. Three subsequent washes in PBS removed unbound secondary antibodies. Cell nuclei were stained using propidium iodide and incubated for 5 minutes. Excess stain was washed off with PBS/TWEEN. A drop of DABCO was added to mount the slide.

In the second part of the study, the effects of exposure time to FGF-2 were investigated. Mesenchymal stem cells were exposed to media containing 100 ng/ml FGF-2 for 4, 6, 16, 24 hours. After exposure, the media was changed and cells were cultured for a week. At the end of day 7, the cells were collected by trypsinisation. Cell proliferation assay by ³H-Thymidine incorporation was then performed.
The results of the above mentioned assays were analyzed with one-way analysis of variance (ANOVA) test at the level of significance of $p \leq 0.05$. For ANOVAs with significant F tests, a Tukey’s post-hoc procedure was performed to determine which treatment groups were significantly different from the other groups. The groups which are of different levels of significance will be presented with different Tukey’s letters. Normality of the data was tested by Kolmogorov-Smirnov test using SPSS-9 software and all the data were proved to be suitable for parametric tests.
2.3 RESULTS

Part I: The effects of different concentrations of FGF-2

The results of cell proliferation were shown in Figures 2-1 to Figure 2-4. The results showed that the higher the concentration was, the higher the cell proliferation rate would be. This was shown in all four time points. Dose-dependent effects on cell proliferation were then demonstrated by $^3$H-Thymidine incorporation assay on Days 2, 7, 14 and 19. The numerical data can be found in Appendix II.

Figure 2-1. ANOVA analysis in $^3$H-Thymidine incorporation assay of MSCs treated with different concentrations of FGF-2 at Day 2
Figure 2-2. ANOVA analysis in $^3$H-Thymidine incorporation assay of MSCs treated with different concentrations of FGF-2 at Day 7

Figure 2-3. ANOVA analysis in $^3$H-Thymidine incorporation assay of MSCs treated with different concentrations of FGF-2 at Day 14
Figure 2-4. ANOVA analysis in $^3$H-Thymidine incorporation assay of MSCs treated with different concentrations of FGF-2 at Day 19

The results of alkaline phosphatase expression were shown in Figure 2-5 to 2-8. Alkaline phosphatase was expressed in all groups including the control; but the expression of alkaline phosphatase was not significantly higher in the experimental groups compared to the control group at all four time points. There was no statistically significant difference among the experimental groups either.
CHAPTER 2

Figure 2-5. ANOVA analysis in alkaline phosphatase expression of MSCs treated with different concentrations of FGF-2 at Day 2

Figure 2-6. ANOVA analysis in alkaline phosphatase expression of MSCs treated with different concentrations of FGF-2 at Day 7
Figure 2-7. ANOVA analysis in alkaline phosphatase expression of MSCs treated with different concentrations of FGF-2 at Day 14

Figure 2-8. ANOVA analysis in alkaline phosphatase expression of MSCs treated with different concentrations of FGF-2 at Day 19
In all groups, osteocalcin was not detected.

Type 2 collagen immunostaining at Day 11 showed that Type 2 collagen was expressed in all the experimental groups (Figure 2-9 to Figure 2-12) but not in the control group. (Figure 2-13)

Figure 2-9 Type 2 collagen immunostaining at Day 11 in 0.1 ng/ml group

Figure 2-10 Type 2 collagen immunostaining at Day 11 in 1 ng/ml group
Figure 2-11 Type 2 collagen immunostaining at Day 11 in 10 ng/ml group

Figure 2-12 Type 2 collagen immunostaining at Day 11 in 100 ng/ml group

Figure 2-13 Type 2 collagen immunostaining at Day 11 in the control group
Part II: The effects of different exposure time

The result of cell proliferation assay at Day 7 showed that the longer the exposure time was, the higher the cell proliferation was. Also, differences were all statistically significant. The exposure time-dependent effects of FGF-2 on MSCs was demonstrated (Figure 2-14).

![Day 7 3H-Thymidine incorporation](image)

Figure 2-14. ANOVA analysis in $^3$H-Thymidine incorporation of MSCs treated with different exposure time of FGF-2 at Day 7
2.4 DISCUSSION

It has been demonstrated in this study that short exposure of FGF-2 has dose- and exposure time-dependent effects on proliferation of mesenchymal stem cells. Therefore, it is possible that single local injection of FGF-2 accelerates bone formation by increasing the number of the cells which are responsible for bone formation at the fracture sites, particularly, the chondrocytes which are responsible for endochondral ossification.

Negative results of alkaline phosphatase and osteocalcin combined with production of type 2 collagen indicated that short exposure of FGF-2 caused differentiation of chondrocytes rather than osteoblasts. My study indicated that FGF-2 leads to the differentiation of chondrocytes rather than osteoblasts and bone formation may be increased by increased endochondral ossification. Previous study has shown that FGF-2 is a more potent mitogen for fibroblasts and pre-osteoblasts than for differentiated osteoblasts (McCarthy 1989).

Another possible mechanism for single local injection of FGF-2 on bone regeneration is through increased angiogenesis. It has been suggested that exogenous FGF-2 modulates bone formation in vitro by inhibition of terminal differentiation of
chondrocytes in the growth plate; while angiogenesis and concomitant in vivo events are pivotal in the promotion of rapid bone formation (Leunig 1997). A third possible mechanism for single local injection of FGF-2 to accelerate bone regeneration is through its effect of promotion of the osteogenic activity of bone morphogenetic proteins (Ono 1996). It is indicated in the study that a very small amount of FGF-2 has a strong promotive effect on the osteogenic activity of BMPs. This is possible because BMPs are expressed in the fracture healing processes (Bostrom 1995).

According to the results of my study, short exposure of FGF-2 has no stimulatory effects on osteogenic differentiation of mesenchymal stem cells. It has been reported that the effects of FGF-2 on human neonatal calvarial osteoblastic cells are differentiation stage specific (Debiais 1998). In that study, 0.1-100 μg/ml FGF-2 was added. It was found that FGF-2 slightly stimulates cell growth and reduces the expression of osteoblastic markers in less mature cells; whereas it induced osteocalcin production and matrix mineralisation in more mature cells, indicating that the effects of FGF-2 are differentiation specific and that FGF-2 may modulate osteogenesis by acting at distinct stages of cell maturation. The results of this study are in concert with ours that FGF-2 does not promote osteogenic differentiation of mesenchymal stem cells, which are the earliest stage osteoprogenitor cells.
Expression of Type-2 collagen was demonstrated at day 11 in all concentrations of FGF-2 exposing to the cells for 24 hours. The results suggest that short exposure of FGF-2 to mesenchymal stem cells may be chondrogenic. It has been reported that FGF-2 stimulates articular cartilage enlargement in young rats in vivo (Shida 1996). Recently, it has been shown that a single local injection of 100 μg FGF-2 to a close fracture in rat enhanced the proliferation of chondroprogenitor cells but not the maturation of the chondrocytes or replacement of cartilage by osseous tissues (Nakajima 2001). The authors also concluded that in the healing of close fracture of long bones, exogenous FGF-2 had a capacity to enlarge the cartilaginous calluses but not to induce more rapid healing. The results of this study support our finding that FGF-2 stimulates chondrogenic proliferation but not osteogenic proliferation.

In another recently published article (Shida 2001), it was shown that exogenous FGF-2 resulted in up-regulation of TGF-β in rat epiphyseal chondrocytes in an in vitro tissue culture model. The results of this study also support and may explain our finding that Type 2 collagen is expressed after FGF-2 treatment. The expression of Type 2 collagen in our study may also result from the up-regulation of TGF-β in MSCs, which is caused by FGF-2. Short exposure of FGF-2 may be used as a strategy to promote chondrogenic differentiation of mesenchymal stem cells to chondrocytes.
in cartilage tissue engineering and to promote endochondral ossification in bone healing. Future work is needed.
Chapter Three

EFFECTS OF CHEMOTHERAPY ON
MESENCHYMAL STEM CELLS
3.1 Introduction

3.2 Materials and methods

3.3 Results

3.4 Discussion
CHAPTER 3

3.1 INTRODUCTION

Tumours of the musculoskeletal system include those growths that arise from tissues of mesenchymal and neuroectodermal origins. The mesoderm is the middle germ layer of the embryo and gives rise to the connective tissues, cartilage, bone, blood and lymphatic vessels, muscles (including myocardium), and blood cells. The neuroectoderm gives rise to nerves and their sheaths. Malignant tumours of the musculoskeletal system are called sarcomas.

The commonly seen primary malignant bone tumours are osteosarcoma and Ewing’s sarcoma. Osteosarcoma is a primary malignant bone tumour of mesenchymal origin and Ewing’s sarcoma is a round cell neoplasm that originates from neuroectodermal tissues. They are both most prevalent in the first and second decade of life and are most commonly seen in skeletally immature children. The predilection sites for these tumours are in the lower extremities, in particular, in the distal femoral and proximal tibial metaphyseal areas. Although malignant bone tumours are uncommon tumours, the treatment, however, is difficult. In the past, above-knee or below-knee amputation, depending on the location of the tumour, was the only treatment and the results were universally discouraging as around one fourth of the patients died of pulmonary metastasis (Ward 1994; Putnam and Ross 1995).
CHAPTER 3

With the advances in chemotherapy, the survivorship of the patients with malignant bone tumours has increased. Chemotherapy plays a major role in the treatment of high-grade skeletal neoplasms such as osteosarcoma, Ewing's sarcoma and malignant fibrous histiocytoma in both children and adults. Chemotherapy leads to a significant increase in cure rate compared with only local surgical intervention for the treatment of osteosarcoma (Meyers 1993a). Most patients with osteosarcoma of an extremity have no evidence of dissemination beyond the primary site at the time of diagnosis as judged by bone scan, chest radiography, chest computed tomography and MRI scan.

Before the routine use of chemotherapy, the sole form of treatment for patients with osteosarcoma was amputation. Amputation served to render most patients free of tumours. Unfortunately, in approximately 80% of patients with localised, completely resected osteosarcoma, lung metastasis developed within 6 to 12 months of amputation (Klein 1989). This observation established that osteosarcoma has a high incidence of microscopic metastasis (Gill 1988), spread to lungs before or at the time of diagnosis. Because the major course of mortality in osteosarcoma is pulmonary metastasis, efforts in adjuvant chemotherapy (chemotherapy given after surgical removal of tumors) have been made to treat micrometastasis. In the mid-1980's, the Pediatric Oncology Group (POG) in America performed a randomised trial for patients with localised osteosarcoma in which patients received an amputation or limb salvage
procedure and were then randomized to receive no chemotherapy or an aggressive multi-agent chemotherapy regime (Link 1986). The results showed that 2-year event-free survival (EFS) rate was only 20% in patients with no chemotherapy while patients receiving chemotherapy had 65% two-year EFS. The importance of chemotherapy was well documented.

Besides adjuvant chemotherapy, the effects of neo-adjuvant chemotherapy (chemotherapy given before the operation) were also investigated (Ettinger 1986). Although there is no compelling evidence showing that neo-adjuvant chemotherapy compared with adjuvant chemotherapy improves EFS or facilitates rational choice of chemotherapy agents in the post-operative period (Rosen 1985), neo-adjuvant chemotherapy is extensively used nowadays (Wynendaele and van Oosterom 1999). It is believed that neo-adjuvant chemotherapy causes shrinkage of the tumours and facilitates surgical removal. Also, immediate post-biopsy chemotherapy might provide a rapid attack on presumed micrometastatic disease (Bacci 1998). An additional potential benefit of neo-adjuvant chemotherapy is the ability to use the amount of necrosis induced in the primary tumour as a measure of chemotherapy efficacy (Ferguson and Goorin 2001).
For the chemotherapy regimes, it has been proved in a multi-nation trial held by the European Osteosarcoma Intergroup that a two-drug regime, which comprised of six courses of cisplatin and doxorubicin, was as effective as T10 regime (Souhami 1997), which consisted of at least seven different drugs and a much longer treatment time (Meyers 1993b).

From the viewpoint of pharmacology, cisplatin is a platinum compound, which belongs to nonclassic alkylating agents. It interacts with DNA and other intracellular macromolecules to from a number of discrete lesions, including intra- and inter-strand covalent DNA cross-links, DNA-protein cross-links, and nuclear protein phosphorylation. Intra-strand DNA cross-links appear to produce the major cytotoxic effect. Reactive intermediates of the platinum agents are rapidly bound to plasma protein. Only free (unbound) platinum is cytotoxic. The other drug, doxorubicin, acts by binding to DNA by a process called intercalation, in which the drug inserts into the double helix of the DNA between base pairs, interfering with DNA cleavage and relegation. Although the two-drug regime was associated with less clinical side effects, the adverse effects of this regime on normal skeletal tissues and cells have not been investigated.
A third chemotherapy agent which is occasionally used to treat malignant bone
tumours in Europe is methotrexate. Methotrexate is a structural analog of folic acid. It
inhibits the action of dihydrofolate reductase (DHFR), the enzyme responsible for
converting folates to their active tetrahydrofolate form. When high levels of
methotrexate are present, intracellular tetrahydrofolate pools become depleted,
leading to depletion of DNA precursors and inhibition of DNA synthesis (Ackland
and Schilsky 1987).

Reconstruction of the bone defects after surgical removal of tumours comprises
special problems because of the sizes of the massive defects and also due to the
involvement and resection of soft tissues as well as the complications of a prosthetic
joint replacement which is often required. Custom-made massive prostheses have
facilitated limb salvage reconstruction after resection of the tumours. It has also
rendered a painless, free-mobile joint (Unwin 1993). In particular, with the invention
of extendible prostheses, replacement of bone defects in skeletally immature patients
with growth potential has become possible because the length of the prostheses can be
adjusted along with the rate of growth of the opposite limb to avoid leg length
discrepancy (Schindler 1997; Cool 1997). In the past, bone cement was used for
implant fixation. This type of cemented massive arthroplasty has been shown to

90
provide good medium-term results, but showed some deficiencies in the long-term, particularly in younger patients. The major cause of failure is aseptic loosening and that is related to cement mantle failure (Unwin 1996). In order to further improve the survivorship of the massive prostheses, cementless intramedullary fixation using hydroxyapatite-coating prostheses was investigated. The short-term result was successful (Blunn 2000).

Recently, some success has been reported in using distraction osteogenesis to reconstruct bone defects after resection of bone tumours (Kapukaya 2000, Millett 2000). It is suggested that after tumour removal, reconstruction of bone defects with distraction osteogenesis will develop sufficient strength and durability and this method can be used in growing children (Tsuchiya 1997). However, neo-adjuvant chemotherapy will more or less affect the function of osteoprogenitor cells and affect osteogenesis and this has to be taken into consideration.

In the previous two chapters, the importance of mesenchymal stem cells on bone regeneration has been clearly pointed out. This is also true in bone regeneration around the tumour prostheses, in particular, in cementless prostheses. Fixation of the intramedullary portion of the prosthesis to the surrounding bones, i.e.,
osseointegration of the bone-implant interface, relies on the growth, differentiation and matrix production, requiring differentiation of osteoblasts from osteoprogenitor cells. Neoadjuvant chemotherapy may have some adverse effects on these progenitor cells and there is evidence that chemotherapy influences extracortical tissue formation in an animal diaphyseal segment replacement model (Young 1997). However, little was reported in the literature concerning the toxicity of these commonly used chemotherapy agents for malignant bone tumours on mesenchymal stem cells, which are precursors of osteogenic cells. The purpose of this study was to investigate the effects of the commonly used chemotherapy agent, at their therapeutic concentrations, on the proliferation and osteogenic differentiation of mesenchymal stem cells. We hypothesize that cisplatin, doxorubicin and methotrexate at their therapeutic concentrations, are detrimental to proliferation and osteogenic differentiation of MSCs. We also hypothesize different drugs have different toxicities towards MSCs and these detrimental effects are dose- and exposure-time dependent.
CHAPTER 3

3.2 MATERIALS AND METHODS

Mesenchymal stem cell culture. C3H10T1/2 cells, from a murine embryonic stem cell line, purchased from American Type Culture Collection (Manassas, VA, USA), were used in this study. 1.6 x 10^3 cells were seeded into each well of 96-well plates. Unless otherwise specified, the chemicals used were purchased from Sigma-Aldrich (Dorset, UK). These cells were then cultured in Dulbecco’s Modified Eagles Medium-low glucose added with 10% foetal calf serum, 1% non-essential amino acids, 50units/ml penicillin, 50μg/ml streptomycin and 0.3μg/ml amphotericin. Osteogenic supplements (OS) comprised of 10mM β-glycerophosphate, 50μM ascorbic acid phosphate and 10^{-7}M dexamethasone were added to the medium.

Chemotherapy agents. Cisplatin and doxorubicin were supplied by Pharmacia & Upjohn (Milton Keynes, UK). The recommended dosages of the cisplatin and doxorubicin by the European Osteosarcoma Intergroup, 1mg/Kg of cisplatin and 1.2mg/Kg doxorubicin, was used. Then the concentration of the drugs was decided by dividing the dosage by the respective volume of distribution. Cisplatin at the concentration of 0.002 mg/ml and 0.0026 mg/ml doxorubicin were the concentrations added to cell cultures. Cells were divided into four groups. Cisplatin was added in Group 1, doxorubicin in Group 2, both were added in Group 3 and Group 4 was the
sham control. In each group, cells were exposed for 6, 15 and 24 hours. After exposure to the drugs for the desired time period, media containing drugs were removed and replaced with media containing no drugs. This was changed twice a week. Eight to ten replicates were performed for each exposure time in each group.

Methotrexate (David Bull Laboratories, Mulgrave, Australia) was added to the culture system in five different concentrations because the range of therapeutic dosage is variable. It is generally accepted that the body surface area of a 40Kg individual is 1.73m² (O'Marcaigh and Gilchrist 1997). The concentrations were determined by dividing the dosage by the volume of distribution and the concentrations were, 0.0516mg/ml (600mg/m²body surface area) in Group 5, 0.1032mg/ml (1,200mg/m² body surface area) in Group 6, 0.1548 mg/ml (1,800mg/ m² body surface area) in Group 7, 0.2064 mg/ml (2,400mg/ m² body surface area) in Group 8 and 0.258mg/ml (3,000mg/ m² body surface area) in Group 9. In Group 10, cells which were not pre-treated with osteogenic supplements were seeded and 0.0258 mg/ml (300mg/ m² body surface area) was added. Group 11 was the sham control and consisted of cells pre-treated with osteogenic supplements. (Table 3-1)
Table 3-1 Experimental groups

<table>
<thead>
<tr>
<th>Group No</th>
<th>Drug and equivalent dosage</th>
<th>Pre-treatment with OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cisplatin (1mg/Kg)</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>Doxorubicin (1.2 mg/Kg)</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>Cisplatin (1mg/Kg) + Doxorubicin (1.2mg/Kg)</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>Sham Control</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>Methotrexate (600mg/M^2BSA)</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>Methotrexate (1200mg/M^2BSA)</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>Methotrexate (1800mg/M^2BSA)</td>
<td>Yes</td>
</tr>
<tr>
<td>8</td>
<td>Methotrexate (2400mg/M^2BSA)</td>
<td>Yes</td>
</tr>
<tr>
<td>9</td>
<td>Methotrexate (3000mg/M^2BSA)</td>
<td>Yes</td>
</tr>
<tr>
<td>10</td>
<td>Methotrexate (300mg/M^2BSA)</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>Sham Control</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Cell proliferation assay. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and alamar blue assay were performed on days 1, 7, 14, and 21 in groups 1-4. Day 0 was defined as the time to remove the drugs. For alamar blue assay, 100 μl of alamar blue solution was added into each well. The plates were then returned to the incubator for 4 hours. The absorbance was then read at 570nm, with a reference wavelength of 630nm on a Dynatech plate reader. For MTT assay, MTT solution was made by adding MTT powder (Sigma M2128) into phenol red free DMEM, making the final concentration 0.5mg/ml. 100 μl of MTT solution was added into each well. The plates were then returned to the incubator for 3 hours. The MTT solution was then removed from the wells and 100 μl of dimethyl sulfoxide was added to each well and the absorbance was then read at 570nm, with a reference wavelength of 630nm on a Dynatech plate reader. For group 5-11, cell proliferation assay was performed on days 7, 14 and 21 by alamar blue method as described above.

Osteogenic differentiation. Alkaline phosphatase (Alk-p) assay was performed on day 21 in groups 1-4. For the Alk-p assay, cells were freeze-thawed and cell lysates were collected. Triton-X 100 was used as solubilisation reagent. The absorbance was read at 405nm on a Cobas Bio machine. The results of alkaline phosphatase were normalized by total DNA assay using Hoechst 33258 dye reagent (Rago 1990).
**Morphological assessment.** Scanning electron microscopy (SEM) was performed to investigate the effects of cisplatin and doxorubicin on cell morphology, extracellular matrix production, and cell-cell interactions in Groups 1-4. SEM was performed on day 21. Cells were seeded into the middle two rows of a 24-well plate at a density of 2000 cells per well onto circular Thermanox discs and were immersed in 1ml of medium containing osteogenic supplements. After allowing the cells 48 hours to settle, anti-cancer drugs were added to the appropriate wells for 15 hours. On day 21, the cells were fixed, washed, dehydrated, dried and then mounted onto SEM stubs. They were then sputter coated, giving an 8nm coating, and earthed with silver dagganite. The microscope used to visualise the samples was a Jeol Winesem JSM-35C scanning electron microscope (Zeiss, Germany).

**Statistical analysis.** Absorbance data were normalised to permit the unbiased comparison of plates analysed on different days by expressing individual values as a percentage of the mean of the control group in each plate. One-way analysis of variance (ANOVA) and Tukey's analyses were employed to establish the presence of statistically significant differences between experimental regimens and their respective control regimens at the 99% level of significance.
3.3 RESULTS

Cell proliferation. The results of MTT and alamar blue assays are shown in Tables 3-2 and 3-3, respectively. Although the cell numbers in Group 1 peaked twice throughout the course of the study, the net effect of cisplatin was to cause an increase in cell number, such that it was not significantly different from the control regimen. Cells in Groups 2 and 3 immediately reduced in number upon exposure to anti-cancer drugs and remained stagnant for the remainder of the study. This was attributed to the toxic nature of the drug doxorubicin, the net effect of which was to cause a reduction in cell number such that it was significantly different from the control regimen. Cells under the control regimen expanded in number throughout the course of the experiment. In some plates, however, the cell number began to fall due to a lack of sufficient space for cell growth.

Osteogenic differentiation. The results of Alk-p assay were shown in Table 3-4. Cells in Groups 1, 2 and 3 produced insignificant quantities of the enzyme alkaline phosphatase throughout the course of the study. Therefore, the net effect of cisplatin and doxorubicin was to inhibit the production of alkaline phosphatase such that the quantities of alkaline phosphatase in Groups 1, 2 and 3 were significantly different from the control regimen. Cells in Group 4 produced relatively large amounts of
alkaline phosphatase, thus indicating that they were capable of osteoblastic
differentiation.

SEM analysis. SEM images of Groups 1-4 on day 21 were shown in Figure 3-1. A, B, 
C and D give an impression of cell density, size and morphology on day 21 of the 
study for groups 1, 2, 3 an 4 respectively. Image A shows that although there are 
cells under regimen 1, there are fewer of them, resulting in fewer cell-cell interactions 
and therefore no changes in morphology. Images B and C show cell debris only, 
indicating that all cells under these regimens have died. Image D shows that cells 
have expanded rapidly and are in closer proximity to one another, resulting in more 
cell-cell interactions and hence changes in cell morphology. Cells in the top 
right-hand corner of image D have adopted an elongated morphology, which is 
characteristic of fibroblast-like cells whereas cells in the bottom right-hand corner 
have adopted morphology more characteristic of osteoblast-like cells.
Table 3-2. Results of MTT assay in Groups 1-4

<table>
<thead>
<tr>
<th>Group</th>
<th>Exposure Time</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6 hours</td>
<td>0.187 ± 0.041</td>
<td>0.739 ± 0.032 *</td>
<td>1.056 ± 0.112 *</td>
<td>0.638 ± 0.114</td>
</tr>
<tr>
<td></td>
<td>15 hours</td>
<td>0.142 ± 0.004 *</td>
<td>0.282 ± 0.019 *@</td>
<td>0.282 ± 0.119 *@</td>
<td>0.540 ± 0.338</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>0.153 ± 0.011 @</td>
<td>0.259 ± 0.017 *@</td>
<td>0.326 ± 0.154 *@</td>
<td>0.390 ± 0.238 *</td>
</tr>
<tr>
<td>2</td>
<td>6 hours</td>
<td>0.051 ± 0.028 *</td>
<td>0.001 ± 0.002 *</td>
<td>0.003 ± 0.007 *</td>
<td>0.004 ± 0.005 *</td>
</tr>
<tr>
<td></td>
<td>15 hours</td>
<td>0.097 ± 0.030 @</td>
<td>0.000 ± 0.000 *</td>
<td>0.001 ± 0.001 *</td>
<td>0.002 ± 0.003 *</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>0.050 ± 0.019 *</td>
<td>0.003 ± 0.003</td>
<td>0.001 ± 0.003</td>
<td>0.002 ± 0.002</td>
</tr>
<tr>
<td>3</td>
<td>6 hours</td>
<td>0.044 ± 0.029 *</td>
<td>0.001 ± 0.002 *</td>
<td>0.000 ± 0.001 *</td>
<td>0.001 ± 0.002 *</td>
</tr>
<tr>
<td></td>
<td>15 hours</td>
<td>0.095 ± 0.030 @</td>
<td>0.000 ± 0.000 *</td>
<td>0.000 ± 0.001 *</td>
<td>0.001 ± 0.001 *</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>0.037 ± 0.012 *</td>
<td>0.000 ± 0.000 *</td>
<td>0.000 ± 0.000</td>
<td>0.001 ± 0.002 *</td>
</tr>
<tr>
<td>4</td>
<td>6 hours</td>
<td>0.182 ± 0.017</td>
<td>0.871 ± 0.073</td>
<td>0.878 ± 0.092</td>
<td>0.663 ± 0.133</td>
</tr>
<tr>
<td></td>
<td>15 hours</td>
<td>0.095 ± 0.017</td>
<td>0.814 ± 0.076</td>
<td>0.929 ± 0.072</td>
<td>0.519 ± 0.168</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>0.137 ± 0.017</td>
<td>0.762 ± 0.074</td>
<td>0.833 ± 0.147</td>
<td>0.654 ± 0.121</td>
</tr>
</tbody>
</table>

* p<0.01
@ signifies that in each group, the difference between a 6hour plate and its respective 15 and 24hour plates are statistically significant (p<0.01).
Table 3-3. Results of alamar blue assay in Groups 1-4

<table>
<thead>
<tr>
<th>Group</th>
<th>Exposure Time</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6 hours</td>
<td>0.047 ±</td>
<td>0.214 ±</td>
<td>0.175 ±</td>
<td>0.136 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.005 *</td>
<td>0.010 *</td>
<td>0.006 *</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>15 hours</td>
<td>0.034 ±</td>
<td>0.122 ±</td>
<td>0.092 ±</td>
<td>0.153 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.009 *®</td>
<td>0.016 *®</td>
<td>0.037 *®</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>0.037 ±</td>
<td>0.138 ±</td>
<td>0.070 ±</td>
<td>0.143 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.011</td>
<td>0.016 *®</td>
<td>0.011 *®</td>
<td>0.027</td>
</tr>
<tr>
<td>2</td>
<td>6 hours</td>
<td>0.025 ±</td>
<td>0.020 ±</td>
<td>0.017 ±</td>
<td>0.026 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.005 *</td>
<td>0.008 *</td>
<td>0.007 *</td>
<td>0.015 *</td>
</tr>
<tr>
<td></td>
<td>15 hours</td>
<td>0.015 ±</td>
<td>0.010 ±</td>
<td>0.023 ±</td>
<td>0.020 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.008 @</td>
<td>0.005 *@</td>
<td>0.005 *</td>
<td>0.006 *</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>0.019 ±</td>
<td>0.019 ±</td>
<td>0.021 ±</td>
<td>0.019 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.006</td>
<td>0.008 *</td>
<td>0.007 *</td>
<td>0.005 *</td>
</tr>
<tr>
<td>3</td>
<td>6 hours</td>
<td>0.021 ±</td>
<td>0.016 ±</td>
<td>0.017 ±</td>
<td>0.019 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.005 *</td>
<td>0.009 *</td>
<td>0.010 *</td>
<td>0.009 *</td>
</tr>
<tr>
<td></td>
<td>15 hours</td>
<td>0.016 ±</td>
<td>0.005 ±</td>
<td>0.016 ±</td>
<td>0.018 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.008</td>
<td>0.003 *®</td>
<td>0.008 *</td>
<td>0.008 *</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>0.017 ±</td>
<td>0.015 ±</td>
<td>0.018 ±</td>
<td>0.014 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.008 *</td>
<td>0.006 *</td>
<td>0.003 *</td>
<td>0.006 *</td>
</tr>
<tr>
<td>4</td>
<td>6 hours</td>
<td>0.036 ±</td>
<td>0.232 ±</td>
<td>0.137 ±</td>
<td>0.141 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.003</td>
<td>0.014</td>
<td>0.040</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>15 hours</td>
<td>0.016 ±</td>
<td>0.181 ±</td>
<td>0.166 ±</td>
<td>0.117 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.008</td>
<td>0.016</td>
<td>0.018</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>0.029 ±</td>
<td>0.203 ±</td>
<td>0.147 ±</td>
<td>0.143 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.004</td>
<td>0.010</td>
<td>0.008</td>
<td>0.025</td>
</tr>
</tbody>
</table>

*p<0.01

@ signifies that in each group, the difference between a 6hour plate and its respective 15 and 24hour plates are statistically significant (p<0.01).
### Table 3-4. Results of alamar blue assay in Groups 5-11

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.148 ± 0.01</td>
<td>0.204 ± 0.03@</td>
<td>0.206 ± 0.04</td>
<td>0.297 ± 0.03@</td>
</tr>
<tr>
<td>6</td>
<td>0.146 ± 0.01</td>
<td>0.191 ± 0.04</td>
<td>0.233 ± 0.06</td>
<td>0.293 ± 0.03@</td>
</tr>
<tr>
<td>7</td>
<td>0.147 ± 0.01</td>
<td>0.200 ± 0.02@</td>
<td>0.215 ± 0.07</td>
<td>0.296 ± 0.02@</td>
</tr>
<tr>
<td>8</td>
<td>0.139 ± 0.02</td>
<td>0.187 ± 0.04</td>
<td>0.222 ± 0.06@</td>
<td>0.283 ± 0.02@</td>
</tr>
<tr>
<td>9</td>
<td>0.145 ± 0.02</td>
<td>0.170 ± 0.05</td>
<td>0.222 ± 0.06@</td>
<td>0.281 ± 0.02@</td>
</tr>
<tr>
<td>10</td>
<td>0.03 ± 0.008**</td>
<td>0.052±0.0081**</td>
<td>0.033±0.0076**</td>
<td>0.029±0.0084**</td>
</tr>
<tr>
<td>11</td>
<td>0.147 ± 0.01</td>
<td>0.174 ± 0.05</td>
<td>0.231 ± 0.06@</td>
<td>0.294 ± 0.01@</td>
</tr>
</tbody>
</table>

* signifies that the cell number is statistically higher in comparison to the control group (p<0.01)
** signifies that the cell number is statistically lower in comparison to the control group (p<0.01)
@ signifies that the cell number, within a single experimental group, is statistically greater than in the previous week (p<0.01).
## Table 3-5 Results of alkaline phosphatase assay in Groups 1-4

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Exposure Time</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6 hours</td>
<td>$0.178 \pm 0.355^*$</td>
</tr>
<tr>
<td></td>
<td>15 hours</td>
<td>$1.945 \pm 2.143^*$</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>$0.193 \pm 0.328^*$</td>
</tr>
<tr>
<td>2</td>
<td>6 hours</td>
<td>$5.125 \pm 5.309^*$</td>
</tr>
<tr>
<td></td>
<td>15 hours</td>
<td>$0.000 \pm 0.000^*$</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>$0.000 \pm 0.000^*$</td>
</tr>
<tr>
<td>3</td>
<td>6 hours</td>
<td>$2.745 \pm 2.547^*$</td>
</tr>
<tr>
<td></td>
<td>15 hours</td>
<td>$4.535 \pm 2.933^*$</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>$0.608 \pm 0.790^*$</td>
</tr>
<tr>
<td>4</td>
<td>6 hours</td>
<td>$15.360 \pm 4.183$</td>
</tr>
<tr>
<td></td>
<td>15 hours</td>
<td>$15.763 \pm 4.989$</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>$14.223 \pm 2.987$</td>
</tr>
</tbody>
</table>

* $p < 0.01$
CHAPTER 3

Figure 3-1 (A). SEM image of MSCs in group 1 on day 21 (Mag x 200)

Figure 3-1 (B). SEM image of MSCs in group 2 on day 21 (Mag x200)

Figure 3-1 (C). SEM image of MSCs in group 3 on day 21 (Mag x200)

Figure 3-1 (D). SEM image of MSCs in group 4 on day 21 (Mag x200)
3.4 DISCUSSION

It has been demonstrated in this study that cisplatin and doxorubicin significantly inhibited proliferation and osteogenic differentiation of mesenchymal stem cells in tissue culture. There is evidence in the literature that chemotherapy not only suppresses haematopoietic stem cells in the bone marrow but also influences stromal cells (Dahl 1983, Papadopoulou and Tsiftsoglou 1996, Guest and Uetrecht 2000). Also, it has been proved that the gene expression of bone marrow stromal cells was modulated by chemotherapy in cells obtained from the patients post chemotherapy and by in vitro treatment (Schwartz 2000). Our findings further support results of previous studies, showing that chemotherapy agents do affect bone marrow stromal cells.

On the other hand, methotrexate did not inhibit the proliferation of MSCs when they were grown in culture with ascorbic acid phosphate, dexamethasone and β-glycerophosphate (osteogenic supplements) but inhibited the growth cells in culture without osteogenic supplements. Methotrexate is known to affect bone metabolism and histomorphometry in rats (May 1994). Methotrexate has been shown to reduce experimental heterotopic new bone formation induced by demineralised bone matrix in rats (Nilsson 1984, Nilsson 1987, Nilsson 1990). Clinically, long-term use of
high-dose methotrexate is associated with loss of bone mass (Gnudi 1988). Loss of bone mass caused by long-term methotrexate is not observed in patients receiving low-dose treatment (Bianchi 1999, Carbone 1999). In an in vitro study (Uehara 2001), it has been shown that methotrexate inhibited the expression of alkaline phosphatase as well as formation of calcified nodules but not proliferation of both MC3T3-E1 (mouse osteogenic cell line) and SaOS-2 (human osteoblastic osteosarcoma cell line) cells. The authors suggested that methotrexate suppresses bone formation by inhibiting the differentiation of early osteoblastic cells. We found that methotrexate affects both proliferation and differentiation of MSCs but not OS-treated, differentiated cells. It is possible that after the treatment of osteogenic supplements, MSCs become committed and differentiated, so that they can resist the detrimental effects of methotrexate and that is the reason why neither proliferation nor osteogenic differentiation is affected. It would be interesting to know whether MSCs treated with osteogenic growth factors (BMP-2, OP-1) can also resist the detrimental effects of methotrexate. Further studies are required.

The findings are important because during limb salvage surgeries, MSCs are responsible for osseo-integration at bone-implant interface. This implies that for these tumour prostheses, it may take longer to achieve sound fixation periprosthetically
because of neo-adjuvant chemotherapy. Also, I suggest that during the postoperative adjuvant chemotherapy, special attention must be paid to protect the implant from loading leading to motion at the interface, which in an adverse environment may promote the formation of fibrous membrane rather than osseo-integration.

Since the toxicity of cisplatin and doxorubicin has been demonstrated in this study, achieving early osseo-integration using tissue engineering methods is a reasonable approach. For example, mesenchymal stem cells can be harvested before the start of neo-adjuvant chemotherapy, these cells can be cultured and expanded during the course of neo-adjuvant therapy. They can then be applied at the bone-implant interface at the time of the operation to facilitate quicker osseo-integration, so that the longevity of the implants can be increased.

Using culture-expanded mesenchymal stem cells harvested before chemotherapy, or allogenic mesenchymal stem cells to accelerate osseo-integration might be a new approach to shorten healing time, allow earlier weight bearing as well as to increase the longevity of implant fixation of these tumour prostheses. Recently, there is evidence that mesenchymal stem cells are immune-privileged (Klyushnenkova 1998). The other possible strategy is to up-regulate the expression of multiple drug resistance
(MDR-1) gene (Takeshita 1996, Scotlandi 1999, Takeshita 2000) of exogenous MSCs or to use genetically modified MSCs which express MDR-1 gene so that these chemotherapy drugs can selectively react on target malignant cells. Safety, however, is the most important consideration. It imperative that MSCs do not undergo malignant transformation after any genetic modification or up-regulation of MDR-1 gene. Lastly, it would be useful to investigate the effects of pre-treatment of MSCs with growth factors such as BMP-2 and OP-1 on the resistance of the detrimental effects of cisplatin and doxorubicin.
Chapter Four

EFFECTS OF CHEMOTHERAPY ON ADULT AND IMMATURE RAT SKELETON
4.1 Introduction

4.2 Materials and methods

4.3 Results

4.4 Discussion
CHAPTER 4

4.1 INTRODUCTION

Chemotherapy has significantly increased the survival rate of patients with malignant bone tumours (Goorin and Andersen 1991, Winkler 1991). Among these patients, a number are skeletally immature. Because of the systemic administration of these drugs, bone cells and growth plates are exposed to them. There is evidence in the literature that these drugs are toxic to osteoblasts and growth plate chondrocytes (Friedlaender 1984, Wheeler 1995a, Bar-On 1993, Robson 1998). In my study, it has been shown that chemotherapy drugs reduce cell proliferation and osteogenic differentiation in MSCs (Chapter 3). Also, it has been shown that chemotherapy has negative effects on the longitudinal growth in skeletally immature individuals (Wollner 1980, Glasser 1991, Davies 1994).

In order to minimise the side effects and complications of chemotherapy on normal bone tissues, an intensive two-drug chemotherapy regime, in which only cisplatin and doxorubicin were used, was developed by European Osteosarcoma Intergroup (Souhami 1997). The results of using cisplatin and doxorubicin in operable osteosarcoma were shown to be as effective as lengthy, multi-drug T10 regimen (Meyers 1992). This two-drug regimen is shorter in duration and is better tolerated by the patients. Additionally, the cost of drugs in the two-drug regimen is substantially
lower. The effects of cisplatin and doxorubicin on the mechanical properties of bones and growth plates are not clear either. It has been reported that growth arrest is associated with chemotherapy in patients with malignant bone tumours, and growth arrest line at the growth plates are visible radiologically in skeletally immature patients with malignant bone tumours years after chemotherapy treatment (Ogden 1984, Cool 1997). Therefore, the purpose of this study is to investigate the effects of systemic chemotherapy on the mechanical properties of adult and skeletally immature bones. We hypothesize that systemic chemotherapy with cisplatin and doxorubicin has detrimental effects on growth plates and the mechanical strength of long bones.
4.2 MATERIALS AND METHODS

Twenty-four immature Wistar rats at the age of 3-5 weeks and the weight between 90 and 110g, and twelve adult Wistar rats at the age of 12 months and the weight of 600g, were used in this study. They were equally divided into two groups so each group consisted of 12 immature and six adult rats. In the experimental group, cisplatin (1mg/Kg body weight) and doxorubicin (1.2mg/Kg body weight), the two-drug chemotherapy regimen suggested by European Osteosarcoma Intergroup, were given to the rats intraperitoneally. In patients, these chemotherapy drugs are given via central venous lines because they can cause vasculitis if they are given via peripheral veins. The reason to give the chemotherapy drugs to the rats intraperitoneally in my experiment is that it would have been technically very difficult to give the drugs to the rats via central venous lines as this is extremely difficult. It is not sensible to administer the chemotherapy drugs via tail veins either as this can cause vasculitis.

Three injections were given. The interval between each injection was two weeks. In the control group, normal saline of the same amount was given intraperitoneally. Seven days after the third injection, which was five weeks after the first injection, the rats were killed. Both femora in each animal were harvested and all the soft tissues surrounding the bones were removed. Mechanical testing was performed using a material testing machine (Houndsfield, Redhill, United Kingdom) (Figure 4-1). For
six adult and six immature rats in each group, a four-point bending load-to-failure test was performed on one femur to measure the ultimate bending strength under the loading rate of 50 Newtons per second (Figure 4-2). Torsion load-to-failure was performed on the other femur to determine the ultimate torsional strength under the loading rate of 5 Newtons per second (Figure 4-3 and 4-4). The specimens were embedded with bone cement in the testing rig. Sagittal sections of the proximal tibial growth plates were prepared by the methods reported in the literature (Brighton 1987). Briefly, the specimens of retrieved tibiae were fixed in 10% formal saline, decalcified in EDTA, dehydrated in an alcohol series, embedded in resin, sectioned using a sledge microtome, and stained with hematoxylin and eosin. Numbers of proliferative and hypertrophic chondrocytes within each column of the growth plates were determined. Ten to 15 columns of cells were counted and averaged in each growth plate section. In the remaining 6 immature rats in each group, the shear load needed to remove the distal femoral epiphysis from the diaphysis was measured under the loading rate of 10 Newtons per second (Figure 4-5). The results of histological analysis and mechanical testing were then analyzed and compared using Student-T tests at $p=0.05$. Normality of the data was justified by Kolmogorov-Smirnov tests performed on SPSS-9 software and all the data were proved to be suitable for parametric tests.
Figure 4-1. Setup of material testing machine for four-point bending test

Figure 4-2. Testing rig for four-point bending test
Figure 4-3. Testing Rig for ultimate torsion test

Figure 4-4. Bone fractured under torsional load

Figure 4-5 Separation of distal femur physis under shear load
4.3 RESULTS

The body weights of the rats are shown in Table 4-1. The body weight of the adult rats in the control group was 614.7±43.9 grams (mean±S.D) and 598.9±50.6 grams in the chemotherapy group. The body weight of the immature rats was 130.5±22.8 grams in the control group and 126.0±17.2 grams in the chemotherapy group. There is no statistically significant difference in body weights between the control and the experimental group at the time of sacrificing the rats. The results of ultimate bending strength are shown in Table 4-2. The ultimate bending strength of the adult rats in the control group was 277.2 ± 22.7 newtons and 222.0 ± 51.2 newtons in the chemotherapy group (p = 0.018). An example of load-deformation curve of the four-point bending test is shown in Figure 4-6. The ultimate bending strength of the immature rats was 131.7 ± 54.9 newtons in the control group and 91.8 ± 16.1 newtons in the chemotherapy group (p=0.061). The results of ultimate torsional strength are shown in Table 4-3. The ultimate torsional strength of the adult rats in the control group was 10.4 ± 2.5 newtons and was 7.4 ± 2.2 newtons in the chemotherapy group (p=0.026). The ultimate torsional strength of the immature rats was 11.0 ± 1.7 newtons in the control group and 9.2 ± 1.9 newtons in the chemotherapy group (p=0.056). Both ultimate bending and torsional strength reduced significantly in the chemotherapy group in adult rats and marginally reduced in the immature rats. The
results of ultimate shear strength of the distal femoral physis are shown in Table 4-4. The ultimate shear strength of the distal femoral physis was $45.8 \pm 9.7$ newtons in the control group and was $35.8 \pm 8.5$ newtons in the chemotherapy group ($p=0.044$). The ultimate shear strength of the distal femoral physis was reduced significantly in the chemotherapy group. An example of load-deformation curve of the ultimate shear strength test is shown in Figure 4-7. Histology of the growth plates showed that in the control group, there were more proliferative and hypertrophic chondrocytes (Figure 4-8). However, in the chemotherapy group, the numbers of proliferative and hypertrophic chondrocytes reduced and the cells were smaller (Figure 4-9). It was also noted that the width of the growth plate was thinner in the chemotherapy group than in the control group. The growth plates in the chemotherapy group are less organized and the cell columns are less well aligned. The septums among the cell columns in the chemotherapy group are also thinner. The numbers of proliferative and hypertrophic chondrocytes are shown in Table 4-5. The average number of proliferative chondrocytes in the control group was $17.0 \pm 3.8$ and $11.8 \pm 1.8$ in the chemotherapy group ($p < 0.001$). The average number of hypertrophic chondrocytes in the control group was $11.2 \pm 2.1$ and $6.9 \pm 2.0$ in the chemotherapy group ($p < 0.001$). Cell numbers were significantly reduced in the chemotherapy group.
Figure 4-6. An example of load-deformation curve of a four-point bending test in the adult control group
Figure 4-7. An example of load-deformation curve in the test of ultimate shear strength of the distal femoral physis in the control group.
### Table 4-1 Body weight of the rats at the time of sacrifice (g)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Control</th>
<th>Chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>614.7±43.9</td>
<td>598.9±50.6</td>
</tr>
<tr>
<td>Immature</td>
<td>130.5±22.8</td>
<td>126.0±17.2</td>
</tr>
</tbody>
</table>

### Table 4-2 Ultimate bending strength (N)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Control</th>
<th>Chemotherapy</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>277.2 ± 22.7</td>
<td>222.0 ± 51.2</td>
<td>0.018</td>
</tr>
<tr>
<td>Immature</td>
<td>131.7 ± 54.9</td>
<td>91.8 ± 16.1</td>
<td>0.061</td>
</tr>
</tbody>
</table>

### Table 4-3 Ultimate torsional strength (N)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Control</th>
<th>Chemotherapy</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>10.4 ± 2.5</td>
<td>7.4 ± 2.2</td>
<td>0.026</td>
</tr>
<tr>
<td>Immature</td>
<td>11.0 ± 1.7</td>
<td>9.2 ± 1.9</td>
<td>0.056</td>
</tr>
</tbody>
</table>

### Table 4-4. Ultimate shear strength of distal femoral physis (N)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Control</th>
<th>Chemotherapy</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature</td>
<td>45.8 ± 9.7</td>
<td>35.8 ± 8.5</td>
<td>0.044</td>
</tr>
</tbody>
</table>

### Table 4-5. Cell numbers per column in the growth plate

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Control</th>
<th>Chemotherapy</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative</td>
<td>17.0 ± 3.8</td>
<td>11.8 ± 1.8</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hypertrophic</td>
<td>11.2 ± 2.1</td>
<td>6.9 ± 2.0</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*Values in Table 4-1 to Table 4-5 are mean ± S.D.*
Figure 4-8. An example of histology of the growth plate in the control group (Mag x100)

Figure 4-9. An example of the histology of the growth plate in the chemotherapy group (Mag x100)
4.4 DISCUSSION

It has been shown in this study that systemic administration of cisplatin and doxorubicin significantly reduced both the ultimate bending and torsion strength of long bones in both adult and immature rats. It may be due to the impaired osteoblast differentiation resulting from a direct effect of chemotherapy (Glackin 1992; Crofton 2000) and relative osteomalacic new bone formation caused by chemotherapy (Okita and Block 1979). The immature mineralised matrix might account for the reduction of the strength of the bones. It is also possible that the size of bones, particularly in the immature rats, is affected by chemotherapy and reduced thickness of the cortices and cross-sectional area of the femur will also result in reduced ultimate bending and torsion strength. Therefore, it would be useful to treat these patients with the medications that help bone mass formation, such as calcium, vitamin D, calcitonin (Li 1996).

Another possible reason for chemotherapy to reduce the strength of the bones is because the activity level of the rats is reduced after chemotherapy due to general discomfort. It has been demonstrated that exercise, in particular running, plays an important role on the mechanical strength as well as the morphometry and bone mass of the rat skeleton in an experimental model (Wheeler 1995b). It is extremely difficult
to quantitatively evaluate the differences of the activity level of the rats before and after chemotherapy injection, however, it is observed that the activity of the rats reduces for a few days after each chemotherapy injection. Whether this short period of reduced activity will effect bone formation is unclear.

A third possible reason to account for the decreased bone strength is reduced food intake caused by the poor appetite after chemotherapy. It has been proved in animal models that nutrition deficiency significantly influences the strength of the bones (Ferguson 1999). However, there was no significant difference in the average body weight of the rats between the two groups at the time of sacrifice. Therefore, it is difficult to judge whether food intake plays a role in this study.

The results of this study showed that the ultimate shear strength of the growth plate has been significantly influenced by chemotherapy. Also, the numbers of proliferating and hypertrophic chondrocytes were significantly reduced. The results support the in vitro work in the literature (Robson 1998). In that study, it was reported that chemotherapy agents reduced growth plate chondrocyte proliferation in both monolayer and suspension cultures. This resulted from either an increase in cell doubling times with a concomitant reduction in the numbers of S phase cells, or a cell
cycle arrest and subsequent cell death. The altered histological appearance shown in this study also strongly supports the influence of chemotherapy on growth plate chondrocytes. The change of cells and microstructures of growth plates might also account for the reduced mechanical strength, as well as growth retardation and short stature of the patients after chemotherapy (Olshan 1992, Davies 1994). In a similar study in which single chemotherapy drug was given to skeletally immature rats (van Leeuwen 2000), it was reported that doxorubicin but not cisplatin decreased the length growth of rat tibia. The authors of that article stated that the decrease in growth might be a direct effect of doxorubicin and methotrexate on the tibial growth plate and metaphysis, but may be more pronounced due to the malnutrition.

The results of this study indicated that for patients receiving two-regimen chemotherapy with cisplatin and doxorubicin, bones are weakened and special attention has to be paid to prevent these patients from injury. Also, weakness of the bones resulting from neo-adjuvant chemotherapy may cause problems when resection and reconstructive surgeries are performed.

The weakness of the growth plates may also increase the risk of physeal injuries in skeletally immature patients undergoing cisplatin and doxorubicin chemotherapy.
Therefore, protection of these patients is important as the chance of sustaining physeal injuries after minor trauma or fall is higher.
Chapter Five

EFFECTS OF CHEMOTHERAPY ON BONE REGENERATION
CHAPTER 5

5.1 Introduction

5.2 Materials and methods

5.3 Results

5.4 Discussion
5.1 INTRODUCTION

Chemotherapy has been used to treat musculoskeletal malignancies. Neo-adjuvant chemotherapy is able to achieve tumour shrinkage and facilitates surgical removal (Ettinger 1986). Neo-adjuvant chemotherapy has been given routinely before resection and reconstruction of malignant bone tumours such as osteosarcoma and Ewing's sarcoma. Cisplatin and doxorubicin are the most commonly used neo-adjuvant chemotherapy drugs for malignant bone tumours in Europe (Souhami 1997). The combined use of cisplatin and doxorubicin has been proved to be as effective as Rosen's T10 regime, which is popular in North America. Rosen's T10 regime involves the use of 10 different chemotherapy drugs and the total course is much longer (Meyers 1992; Postma 1993). Therefore, it is believed that the two-drug regime has far fewer side effects. However, because these drugs are administered via intravenous infusion, they will have systemic adverse effects. A previous section in my thesis has demonstrated the detrimental effects of chemotherapy on cells in vitro and the effects of these agents on growth plate morphology, ultimate strength of the femur and the ultimate shear strength of the physis. In the same way, these drugs might affect the rate of osseo-integration around the tumour prostheses.

Fracture healing model in rats has been used for a long time to evaluate bone
regeneration (Bonnarens and Einhorn 1984). The advantages of using rats to evaluate bone regeneration include short experiment time, easy accessibility of the animals and low costs. To evaluate detrimental effects of chemotherapy agents on osseo-integration and bone regeneration, rat fracture gap healing model can also be used.

The purpose of this study is to investigate the effects of chemotherapy on bone regeneration using an in vivo animal model. The hypothesis of this study is that chemotherapy with cisplatin and doxorubicin reduces the rate of bone regeneration.
5.2 MATERIALS AND METHODS

Twenty-four adult male Wistar rats, with the average body weight of 600g, were used in this study. They were equally divided into two groups. In the experimental group, three courses of systemic chemotherapy with cisplatin (1mg/Kg body weight) and doxorubicin (1.2mg/Kg body weight), the chemotherapy regime suggested by European Osteosarcoma Intergroup (Souhami 1997), were given intraperitoneally. In the control group, normal saline was injected intraperitoneally. Three injections were given. The interval between each injection was two weeks. Seven days after the third injection, the animals were operated. All the operations were performed in the standard animal operation theatre in the Royal Veterinary College. A project license for the use of experimental animals was obtained from the Home Office. The procedures of the operation were as follows. Under inhalation general anaesthesia, the skin over left hind leg was disinfected and draped. A four-centimetre skin incision was made at the lateral side of the left thigh. After the skin incision was made, the underlying muscles were exposed and another incision was made along the intermuscular septum to expose the femoral shaft. After the femoral shaft was adequately exposed, four holes were drilled with a 1.5 mm drill bits, using the plate of the external fixator as a drill guide. The 2.0 mm pins were then screwed into the pre-drilled holes. After the pins were inserted into the bone, four tiny holes were
punched on the superior skin flap and the pins were passed through the holes on the skin flap. The pins were then secured to a plate (Figure 5-1) with epoxy glue Araldite (Bostik Ltd, Leicester, UK). After the glue had set, a one-millimeter osteotomy was made between the second and the third pin. The wound was then closed layer by layer. Antibiotics and analgesics were given after the operation. Six rats in each group were killed 3 weeks after the operation and the other six rats were killed 5 weeks after the operation. Bone mineral density measurement by dual-energy X-ray absorptiometry (DEXA) scan (QDR-1000, Hologic, USA) was performed once every week on rats that were kept for 5 weeks after the operation in each group. Inhalation anaesthesia was given to the rats when performing DEXA scanning. A specially designed mounting device was used to hold the plate of the external skeletal fixator in place in order to make sure the legs were positioned in the same manner during each DEXA scanning (Figure 5-2). Calibration of the DEXA scanning was performed by phantom scanning every week before the scanning of the rats. Radiographs were taken of each animal at the time of sacrifice. After the animals were sacrificed, the left femur was retrieved. The specimens were fixed in 10% formal saline, through a series of alcohols and decalcified in EDTA. After the specimen was decalcified it was dehydrated in an alcohol series, embedded in wax, sections cut using a sledge microtome, and stained with hematoxylin and eosin. Histology of the regenerated
bones was performed and the qualitative analysis was made. The results of weekly DEXA scan between the control and the experimental chemotherapy group were statistically analyzed with Student’s T-test at p=0.01. The normality of all the numerical data was tested by Kormagorov-Smirnov test using SPSS-9 software and all the data were proved to be suitable for parametric tests.

Figure 5-1 A photograph showing the external skeletal fixator used in this study
Figure 5-2 DEXA scanning

Figure 5-3. Mounting device for DEXA scan
5.3 RESULTS

One example of the radiographs in each group is shown in Figure 5-4 to Figure 5-7.

The X-rays in the control group taken three weeks after the operation show that there was some new bone formation in the fracture gaps (Figure 5-4) but the gaps were still clearly visible in the chemotherapy group (Figure 5-5). Besides, there was more periosteal callus formation in the control group than in the chemotherapy group. In the x-rays taken five weeks after the operation, the fractures almost heal and the fracture gaps were almost invisible in the control group (Figure 5-6). However, in the chemotherapy group, the gaps still existed and resorption of the bone ends was noted (Figure 5-7). Again, periosteal callus formation was more abundant in the control group than in the chemotherapy group five weeks after the operation. Histology shows that in the control group, there is abundant osteoid matrix production and bone cells migrating into the gap to fill in the defect (Figure 5-8). However, in the chemotherapy group, there were mostly fibroblasts in the fracture gap and very little bone formation was noted in the fracture gaps (Figure 5-9). DEXA scan results are shown in Figure 5-10. The increase of bone mineral density is slower in the chemotherapy group than in the control group and the bone mineral density in the fracture gap in the chemotherapy group was statistically lower than that in the control group at all five time points from week 1 to week 5.
Figure 5-4. A radiograph showing bone regeneration three weeks after the operation in the control group

Figure 5-5. A radiograph showing bone regeneration three weeks after the operation in the chemotherapy group
Figure 5-6. A radiograph showing bone regeneration five weeks after the operation in the control group

Figure 5-7. A radiograph showing bone regeneration five weeks after the operation in the chemotherapy group
Figure 5-8. Histology of the regenerated tissue in the osteotomy gap in the control group five weeks after the operation (Mag x160)

Figure 5-9. Histology of the regenerated tissue in the osteotomy gap in the chemotherapy group five weeks after the operation (Mag x160)
5.4 DISCUSSION

It has been demonstrated in this study that chemotherapy with cisplatin and doxorubicin delays the rate of bone regeneration in the fracture gap. The results were similar to the previous study in the literature (Seltzer, 1998; Sato, 1999). Adriamycin (doxorubicin) and methotrexate have been shown to reduce the volume of bone regeneration and stiffness of the newly formed bone at the fracture gap in rat osteotomy gaps (Clark, 1997).

Apart from fracture healing and bone regeneration, in the literature it has also been reported that cisplatin alone and delayed addition of chemotherapy reduce the prosthesis at a canine diaphyseal segmental replacement model (Young, 1997) and frozen allogeneic segmental autografts (Zart, 1993). Also, doxorubicin (adriamycin) has been shown to suppress the new bone generation within and around cortical segmental autografts in a canine segmental cortical autograft experiment (Turchildt, 1998). Although cisplatin and doxorubicin is the most popular chemotherapy regime for the treatment of malignant bone tumours, their effects on bone regeneration has not previously been investigated. In my study, the influence of chemotherapy on bone regeneration was quantitatively defined.

Figure 5-10. Bone mineral density in the osteotomy gaps of the rats treated and not treated with chemotherapy (Refer to Appendix II for numerical data)
5.4 DISCUSSION

It has been demonstrated in this study that systemic chemotherapy with cisplatin and doxorubicin delays the rate of bone regeneration in the fracture gap. The results were similar to the previous study in the literature (Pelker 1985), in which adriamycin (doxorubicin) and methotrexate have been shown to reduce the ultimate torsion strength and stiffness of the newly formed bone at the fracture gap in a rat experiment.

Apart from fracture healing and bone regeneration, in the literature it has also been reported that cisplatin alone also delayed the extracortical bone formation around the prosthesis in a canine diaphyseal segmental replacement model (Young 1997). Cisaplatin has also been reported to slow the rate of incorporation of fresh syngenic and frozen allogenic cortical bone grafts (Zart 1993). Also, doxorubicin (adriamycin) has been shown to suppress the new bone formation within and around cortical segmental autografts in a canine segmental cortical autograft experiment (Burchardt 1983). Although cisplatin and doxorubicin is the most popular chemotherapy regime for the treatment of malignant bone tumours, their effects on bone regeneration has not previously been investigated. In my study, the influence of chemotherapy on bone regeneration was quantitatively defined.
Due to the number of variables associated with fracture healing which play an important role in determining the repair and the sequence of cellular events, an animal model is an important tool for fracture healing and bone regeneration studies (Nunamaker 1998). For a long time the rat has been a popular model in that rats are larger than the mice and the mechanical testing is easier. Also, for histology, more specimens are available per animal. Most important of all, the costs of rats are still quite reasonable for large studies (Hietaniemi 1995; Utvag 1994). Therefore, rats were chosen in this study.

The external skeletal fixator used in this study involves the use of super glue to fix the pins to the plate. The advantage of using epoxy glue is that the procedure is simple and quick. However, it is slightly stimulating to the rat skin and it caused superficial skin erosion on two rats. Therefore, in the next section (Chapter 6) the design of the external fixator has been slightly modified by using nuts, one above and one below the plate to secure the pins to the plate and avoid using epoxy glue.

The implication of the results of this study is that in the surgical resection and reconstruction of the bone tumours, impaired bone regeneration may lead to inadequate osseo-integration around the prosthesis and early loosening may occur.
Strategies to improve bone regeneration in these patients, for example, applying bone marrow derived mesenchymal stem cells or growth factors around the prosthesis, need to be developed to increase the longevity of these tumour implants. Special attention needs to be paid to these patients to prevent them from sustaining fractures because fractures in the patients with chemotherapy may be difficult to heal. Therefore, facilitation of bone regeneration in patients receiving chemotherapy with tissue engineered mesenchymal stem cell is an approach that may have potential benefits.
Chapter Six

USE OF MESENCHYMAL STEM CELLS TO
FACILITATE BONE REGENERATION IN NORMAL
AND CHEMOTHERAPY-TREATED RATS
6.1 Introduction

6.2 Materials and methods
   6.2.1 Harvest and culture of rat mesenchymal stem cells
   6.2.2 Culture of rat mesenchymal stem cells in fibrin glue
   6.2.3 Use of mesenchymal stem cells to enhance bone regeneration in normal and chemotherapy-treated rats

6.3 Results
   6.3.1 Culture of rat mesenchymal stem cells
   6.3.2 Survival and proliferation of mesenchymal stem cells in fibrin glue
   6.3.3 Use of mesenchymal stem cells to enhance bone regeneration

6.4 Discussion
6.1 INTRODUCTION

In the previous chapters of my thesis, cisplatin and doxorubicin, the most commonly used chemotherapy drugs for malignant bone tumours, have been demonstrated to have detrimental effects on fracture healing (Chapter 5) and on mechanical strength of the skeleton (Chapter 4). The toxicity of cisplatin and doxorubicin on proliferation and the osteogenic differentiation of mesenchymal stem cells (MSCs) has also been shown (Chapter 3). In patients receiving chemotherapy with segmental bone defects following tumour surgery where reconstruction of the defects is necessary, an osteogenic potential is required. As this study has shown the osteogenic potential may be reduced by systemic chemotherapy, it may therefore be important to apply tissue engineering approaches to facilitate bone regeneration in individuals receiving chemotherapy.

It has been reported that the use of autologous mesenchymal stem cells loaded onto hydroxyapatite scaffolds can successfully heal segmental bone defects in animal models (Kadiyala 1997, Bruder 1998a). In these studies, normal animals without chemotherapy were used; no studies have been published using similar approach to heal bone defects in chemotherapy treated animals. Hydroxyapatite, although having been extensively studied and proved to be a suitable material for bone regeneration,
has its drawbacks. Cells need to be loaded onto hydroxyapatite scaffolds at least a few hours before use. In addition, it is difficult to fill irregularly shaped bone defects with hydroxyapatite.

Fibrin tissue adhesive sealants, sometimes called fibrin glues, have been used for treatment of soft and hard tissue wounds since 1975 (Lambrecht and Klinger 1990). Subsequently, they have been used in conjunction with other biomaterials such as coral or hydroxyapatite to heal bone defects (Kania 1998, Nakamura 1998b). They have also been used to restore small bone defects at craniotomy in conjunction with autologous bone dusts in patients (Matsumoto 1998). Recently, fibrin glue has been introduced to the field of tissue engineering (Horch 1998, Orr 1999, Silverman 2000, Bach 2001); it has been used as scaffolds in musculoskeletal tissue engineering. It has been demonstrated that fibrin glue can be used as a carrier for calvarial osteoprogenitor cells to repair critical-size osseous facial defects (Tholpady 1999) and for periosteal cells to induce heterotopic osteogenesis (Isogai 2000) in rat experiments. It has also been reported that fibrin glue, together with peristeum-derived osteoprogenitor cells, can heal segmental bone defects in rabbits (Perka 2000).

So far, very little can be found in the literature concerning the use of fibrin glue as a carrier for bone marrow-derived mesenchymal stem cells to reconstruct bone defects.
Therefore, it is important to investigate the feasibility of using fibrin glue as a carrier for MSCs. The purpose of this study is to investigate the feasibility of using MSCs from isogenic rats with fibrin glue as a delivery vehicle, to enhance bone regeneration in normal and chemotherapy-treated rats. The hypothesis of this study is that mesenchymal stem cells which are delivered by fibrin glue will increase bone formation at the bone defect sites in normal and chemotherapy-treated rats.
6.2 MATERIALS AND METHODS

6.2.1 HARVEST AND CULTURE OF RAT MESENCHYMAL STEM CELLS

The methods used to harvest and culture bone marrow-derived mesenchymal stem cells from rats were slightly modified from those given in the literature (Maniatopoulos 1988). Bone marrow was harvested from young adult male rats of the Wistar strain, aged 40-43 days and weighing 110-120 g. Rats were killed by cervical dislocation. The skin over the bilateral thigh area was clipped and disinfected with Hydrex (Zeneca, Cheshire, UK). Under aseptic conditions, incisions were made at the lateral sides of the thighs (Figure 6-1). Skin was reflected by blunt dissection. The major muscles around the femur were dissected away (Figure 6-2). The femora were removed, cleaned of soft tissues, and put in the transport media for an hour. The transport media consisted of DMEM plus 10% foetal calf serum, Penicillin G 500u/ml, Streptomycin 500 μg/ml and Amphotericin B (Fungizone) 3 μg/ml. After immersing the bones in the transport media for one hour, containers were opened under a tissue culture hood and the femur bones removed. Both ends of the femoral bone were removed using a sterile scalpel. The marrow was flushed out with 5 ml of defined medium consisting of DMEM-low glucose plus 10% foetal calf serum, Penicillin G 50 u/ml, Streptomycin 50 μg/ml and Amphotericin B (Fungizone) 0.3 μg/ml and was loaded in a 10ml syringe with a 20 gauge needle. The released cells
collected in a 25cm$^2$ culture flask after being suspended by repeated aspiration and expulsion from the syringe and needle. The flask was then placed into an incubator at 37°C, 95% air and 5% CO$_2$. Media was changed after 4 days and non-adherent cells were removed together with the media. Media was then changed twice a week thereafter. After 10-14 days of primary culture, the cells reached confluence and were passaged by using a cell scraper (Falcon 3086) to mobilise the cells. The cells were centrifuged and washed twice. Cell counts were made at this stage. On average, one 25 cm$^2$ flask contained 0.8-2.5 x 10$^5$ cells. Cells were then passaged about every 7-8 days. Passage 2 and passage 3 cells were used for assays.
Figure 6-1. A photograph showing lateral incision for femoral bone exposure

Figure 6-2. A photograph showing that soft tissues were removed and the femur bone was retrieved
6.2.2 CULTURE OF RAT MESENCHYMAL STEM CELLS IN FIBRIN GLUE

One hundred thousand cells were suspended in 1.0 ml of the thrombin component of the fibrin glue (Tisseel®, Baxter Hyland Immuno, UK) (Figure 6-3) before mixing the thrombin and fibrin component. Mixing was carried out in wells of 24-well tissue culture plate by injecting 0.2 ml of each component using the specially designed injection apparatus supplied by the manufacturer (Figure 6-4). Therefore, each 0.4 ml glue mass contained 2x10^4 cells. After the glue had set, culture media was added and cells were cultured at 37°C with 5% CO₂. Alamar blue assay was performed to evaluate cell viability, the results were expressed as the percentage of absorbance of the control. Cells from the same animal cultured at a level of 2x10^4 cells in each well without glue acted as the control. Analysis was performed at 4, 24, 48, 72 and 96 hours. Cell proliferation was measured by ^3H-Thymidine uptake. The masses of glue containing radio-labelled cells were rinsed in protease inhibitors in water, lyopholised, and were digested with 1% papain and the radioactivity of the samples were measured in a scintillator. The results of ^3H-Thymidine were normalized by total DNA, which was measured using Hoechst 33258 DNA assay kit. Cell proliferation assay was performed on cells after 24, 48, 72 and 96 hours in culture. For these two assays, six specimens were measured in each group. Histology, scanning and transmission electron microscopic images were taken at 24 and 96 hours after glue injection. The
specimens for scanning electromicroscopic (SEM) imaging were prepared by fixing in the buffer consisting of 1.5% glutaldehyde and 0.1 M sodium cacodylate for 48 hours at 4°C. Samples were then washed in 0.2% sodium cacodylate for 1 hour and fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were then washed twice for 5 minutes in 0.2 M sodium cacodylate and placed in 1% tannic acid and 0.05 M sodium cacodylate for 1 hour. Samples were then washed with sodium cacodylate buffer 4 times (2 minutes each) and dehydrated in increasing concentrations of alcohol before air drying in hexamethyl disalazene. Samples were mounted onto electron microscopy stubs. Silver in methyl isobutyl ketone (Acheson electrodag 1415M) was applied to the sample-stub interface. The prepared samples were then coated with Palladium using an Emitech K50 coating apparatus. Microscopy was performed using a Jeol JSM-550LV scanning electron microscope.

The specimens for transmission electron microscopy were prepared using the method described in the literature (Lewinson 1982). The specimens were fixed in 1.5% glutaraldehyde and 0.1 M sodium cacodylate buffer, pH 7.4, at 4 degrees for one hour. Samples were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 hours. This was followed by dehydration in serial alcohols and the specimens were section cut 90nm thick on an LKB ultratome, then embedded in resin. The
specimens were picked up into copper grids, stained with uranyl acetate and lead nitrate, and viewed under a Philips CM 12 transmission electron microscope.

Histology was performed on thick (1µm) sections and stained using toluidine blue.

The results of cell viability and cell proliferation assays were statistically analysed using a one-way analysis of variance (ANOVA) to compare values between the four time periods. For ANOVAs with significant F tests, a Tukey’s post-hoc procedure was performed to determine which treatment groups were significantly different from each other. The level of significance was \( p \leq 0.05 \). Normality of the data was tested by Kolmogorov-Smirnov tests using SPSS-9 software and all the data were proved to be suitable for parametric tests.
Figure 6-3. Tisseel® kit

Figure 6-4 Injection apparatus of Tisseel®
CHAPTER 6

6.2.3 USE OF MESENCHYMAL STEM CELLS TO ENHANCE BONE REGENERATION IN NORMAL AND CHEMOTHERAPY-TREATED RATS

A license for the use of rats in this experiment was obtained from the Home Office of the United Kingdom. Thirty-six adult male Wistar rats, at the average weight of 600 grams, were used in the study. Eighteen rats (chemotherapy group) received three intraperitoneal injections of combined cisplatin (1mg/Kg body weight) and doxorubicin (1.2 mg/Kg body weight), the neoadjuvant regime suggested by the European Osteosarcoma Intergroup (Souhami 1997). The interval between the injections was two weeks. Normal saline was injected intraperitoneally to the other 18 rats (control group). Seven days after the last injection, the rats were operated. A 1.5 mm bone gap osteotomy was created on the left femoral shaft and external skeletal fixators were used to stabilize the bone ends. The fixator was similar to the one used previously, with two pins at each side of osteotomy and each pin was connected to the fixator with two nuts, one on each side of the plate. (Figure 6-5). Eighteen rats in each group were divided into three subgroups; there were a) subgroup in which the osteotomy remained unfilled; b) subgroup with fibrin glue filling in the defect, and c) subgroup using MSCs suspended in fibrin glue to fill in the defect. The study groups were numbered as follows: Group 1, normal rats with nothing in the osteotomy gaps; Group 2, normal rats with fibrin glue only in the gaps; Group 3, normal rats with MSCs loaded in fibrin glue in the gaps; Group 4, chemo rats with nothing in the gaps;
6.3.2 SURVIVAL AND PROLIFERATIONS OF MESENCHYMAL STEM CELLS IN FIBRIN GLUE

The results of alamar blue assay were shown in Figure 6-8. In all groups there was around 80% of cell viability compared with control cultures. There were no statistically significant differences in cell viability among different groups. The results of $^3$H-Thymidine incorporation assay were shown in Figure 6-9. Cell proliferation rate was similar in all groups, about 20 CPM/µgDNA/24 hours. There was no significant difference in cell proliferation rates. The SEM image of MSCs in the fibrin glue for 24 hours and 96 hours were shown in Figures 6-10 and 6-11, respectively. It was noted that MSCs were spherical in shape after 24 hours and they became fibroblast-like and developed processes subsequently. This was also shown in the histology (Figures 6-12 and 6-13). Dividing MSCs were noted in the histology (Figure 6-14). TEM images were shown on Figures 6-15 and 6-16. Healthy, dividing cells were noted in both 24-hour and 96-hour specimens. These cells at 24 hours appeared to be round in section indicating a spherical morphology. They are surrounded by a fine electron dense fibrillar material which is the fibrin glue. At 96 hours they were oval in cross section indicating a more flattened morphology at this later stage.
Group 5, chemo rats with fibrin glue only in the gap; Group 6, chemo rats with MSCs loaded in fibrin glue in the gaps.

MSCs were harvested from the femora of skeletally immature male Wistar rats as described previously. Briefly, under sterile conditions, the ends of the femur were removed and the marrow content was flushed out with culture media containing Dulbecco's Modified Essential Medium (DMEM) with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. The flushed marrow content in the media was collected by centrifugation. The cell pellet was re-suspended, washed, and cultured in 175 cm$^2$ culture flasks. Media was changed twice a week. The cells which adhered to the plastic reached confluence at 10-14 days after initial seeding and were passaged after detachment from the flask surface using trypsin. Passage 2 and passage 3 cells were transplanted to the bone defects. One hundred thousand cells were then suspended in 0.25ml of thrombin component of the fibrin glue (Tisseel®, Baxter Hyland Immuno, UK) immediately before mixing with 0.25ml of fibrin component. 0.5 ml of fibrin glue was injected to the gap. Rats were kept for 5 weeks after the operation. Evaluation of bone formation included radiography, histology as well as quantitative histological analysis for percentage bone formation using an image analysis system (KS-300, Zeiss, Germany) and bone mineral density measurement at 156
the osteotomy gap using dual energy X-ray absorptiometry (DEXA) scanning (Hologic QDR-1000, USA) three and five weeks after the operation.

Histology of the specimen was performed as described previously except specimens were embedded in LR white methyl methacrylate resin. After fixing the specimens and dehydrating in an alcohol series the specimens were transferred to a 50%-50% resin alcohol mixture for 3 days and then into pure resin for a further 3 days. The specimens were then embedded in resin and sections (0.3mm thick) were cut on an EXACT band saw, attached to Perspex slides and ground down to 30 μm on an EXACT micro-grinding machine before being polished with aluminum oxide paste. The slides were then stained with Toluidine blue and Paragon. The percentage bone formation at the osteotomy gap was then measured under the image analysis system. The area which was filled with newly formed mineralised tissues in the osteotomy gap was marked manually and the image analysis automatically calculated the value of the area. The value was then divided by the total surface area of the gap, which was also manually defined in the image analysis system. The result obtained was percentage bone formation.

DEXA scanning was performed using the same method described in Chapter 5. Briefly, under inhalation anaesthesia, the left hind limbs of the rats were positioned
using the specially designed rig to ensure the same positioning of all the rats. The bone mineral density at the osteotomy gap area was then performed. Calibration of the DEXA machine was performed by phantom scanning before each lot of testings.

The results of quantitative histological analysis and DEXA scanning were analyzed with one-way analysis of variance (ANOVA) test at the level of significance of $p \leq 0.05$. For ANOVAs with significant F tests, a Tukey's post-hoc procedure was performed to determine which treatment groups were significantly different from each other. Normality of the data was confirmed by a Kolmogorov-Smirnov test on SPSS-9 software and all the data were proved to be suitable for parametric tests.

Figure 6-5. A photograph showing the external skeletal fixator used in this study
6.3 RESULTS

6.3.1 CULTURE OF RAT MESENCHYMAL STEM CELLS

The morphology of rat MSCs in the culture flask under light microscope (LM) and scanning electron microscope (SEM) are shown (Figures 6-6 and 6-7). It was noted that these MSCs had a flat, fibroblast-like morphology on tissue culture plastic.

Figure 6.6 Rat mesenchymal stem cells (Paasage 1, Mag x100)

Figure 6-7. SEM image of rat mesenchymal stem cells (Mag x330)
6.3.2 SURVIVAL AND PROLIFERATIONS OF MESENCHYMAL STEM CELLS IN FIBRIN GLUE

The results of alamar blue assay were shown in Figure 6-8. In all groups there was around 80% of cell viability compared with control cultures. There were no statistically significant differences in cell viability among different groups. The results of \(^3\)H-Thymidine incorporation assay were shown in Figure 6-9. Cell proliferation rate was similar in all groups, about 20 CPM/\(\mu\)gDNA. There was no significant difference in cell proliferation rates. The SEM image of MSCs in the fibrin glue for 24 hours and 96 hours were shown in Figures 6-10 and 6-11, respectively. It was noted that MSCs were spherical in shape after 24 hours and they became fibroblast-like and developed processes subsequently. This was also shown in the histology (Figures 6-12 and 6-13). Dividing MSCs were noted in the histology (Figure 6-14). TEM images were shown on Figures 6-15 and 6-16. Healthy, dividing cells were noted in both 24-hour and 96-hour specimens. These cells at 24 hours appeared to be round in section indicating a spherical morphology. They are surrounded by a fine electron dense fibrillar material which is the fibrin glue. At 96 hours they were oval in cross section indicating a more flattened morphology at this later stage.
Figure 6-8. Alamar blue assay of MSCs in fibrin glue

Figure 6-9. \(^3\)H-Thymidine incorporation assay of MSCs in fibrin glue
Figure 6-10. SEM image of MSCs in fibrin glue for 24 hours (Mag x600)

Figure 6-11. SEM image of MSCs in fibrin glue for 96 hours (Mag x600)
Figure 6-12. Histology of MSCs in fibrin glue for 24 hours (Mag x 600)

Figure 6-13. Histology of MSCs in fibrin glue for 96 hours (Mag x 600)

Figure 6-14. Histology showing dividing MSCs (Mag x 600)
Figure 6-15. TEM image of MSCs in fibrin glue for 24 hours (Mag x20,000)

Figure 6-16. TEM image of MSCs in fibrin glue for 96 hours (Mag x20,000)
6.3.3 USE OF MESENCHYMAL STEM CELLS TO ENHANCE BONE REGENERATION

The body weight of the rats in each group is shown in Table 6-1. There was no significant difference in body weight of the rats among the groups at the time of sacrifice. One X-ray from each group is shown in Figures 6-17 to 6-22. It was noted from the X-rays that there was less bone formation in Group 4 and Group 5 of the chemotherapy-treated rats compared with Group 1 and Group 2, which were normal rats, when there was nothing or only fibrin glue added to the fracture gap. However, bone formation was quite obvious in Group 3 and Group 6, in these groups MSCs were delivered in the fibrin glue. For DEXA scan results (Figures 6-23 and 6-24), statistically significant difference was shown between different groups. Fibrin glue alone did not enhance bone regeneration in either normal or chemotherapy-treated rats as there was no statistically significant difference between Group 1 and 2. In addition, there was no significant difference between Group 4 and 5 at both three weeks and five weeks. Also, it was shown that chemotherapy significantly reduced bone mineral density at the osteotomy gap, as bone mineral density of Group 4 and Group 5 was significantly less than in Group 1 and Group 2 at both time points. However, when the bone defects were filled with fibrin glue and MSCs, significantly greater bone regeneration was noted in Group 3 and Group 6. In these two groups bone mineral density was significantly higher than the other groups at both three-week and
five-week time periods. The result was not different between Group 3 and Group 6, which indicated that bone mineral density at the osteotomy sites of normal and chemotherapy-treated rats were not different if the gaps were filled with MSCs and fibrin glue. This indicated that the introduced stem cells alleviated the effects of chemotherapy.

Histology was shown in Figures 6-25 to 6-30. In Group 1, new bone formation was noted in the gap, but there was little periosteal callus (Figure 6-25). Besides endochondral callus, there was also periosteal callus in Group 2, in which fibrin group was added to the gaps (Figure 6-26). Bone regeneration was evident in Group 3, in which MSCs were delivered to the bone defects with fibrin glue. In the chemotherapy-treated rats, new bone formation was less in Group 4 (Figure 6-28) compared with Group 1 (Figure 6-25). There was also less new bone formation in Group 5 (Figure 6-29) compared with Group 2 (Figure 6-26). Interestingly, Group 5, in which gaps were filled with fibrin glue, had more periosteal callus than Group 4. This finding was in line with that in normal rats as there was more periosteal callus in Group 2 than in Group 1. In Group 6, there was both periosteal and endosteal new bone formation (Figure 6-30) and the amount of new bone formation was comparable to that of normal rats (Group 3, Figure 6-27). The results of quantitative histological
analysis were shown in Figure 6-31. There was no significant difference between Group 1 and Group 2 (38.5% ± 2.6% versus 36.4% ± 3.1%) and they were significantly higher than Group 4 and Group 5 (21.5% ± 4.0% and 23.6% ± 3.8%, respectively). There was no difference between Group 4 and Group 5. Group 3 and Group 6, in which MSCs were delivered, has the highest percentage bone formation (47.7% ± 2.9% and 46.8% ± 3.5%, respectively) and there was no difference between Group 3 and Group 6.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± S.D.)</td>
</tr>
<tr>
<td>1 (Normal control)</td>
<td>627.4 ± 34.9</td>
</tr>
<tr>
<td>2 (Normal + Glue)</td>
<td>643.2 ± 38.5</td>
</tr>
<tr>
<td>3 (Normal + Glue + MSC)</td>
<td>635.9 ± 30.3</td>
</tr>
<tr>
<td>4 (Chemo control)</td>
<td>614.4 ± 40.6</td>
</tr>
<tr>
<td>5 (Chemo + Glue)</td>
<td>607.3 ± 32.0</td>
</tr>
<tr>
<td>6 (Chemo + Glue + MSC)</td>
<td>619.5 ± 51.8</td>
</tr>
</tbody>
</table>

Table 6-1. Body weight of the rats at the time of sacrifice
Figure 6-17. A radiograph showing bone regeneration in Group 1 (Mag x20)

Figure 6-18. A radiograph showing bone regeneration in Group 2 (Mag x20)
Figure 6-19. A radiograph showing bone regeneration in Group 3 (Mag x20)

Figure 6-20. A radiograph showing bone regeneration in Group 4 (Mag x20)
Figure 6-21. A radiograph showing bone regeneration in Group 5 (Mag x20)

Figure 6-22. A radiograph showing bone regeneration in Group 6 (Mag x20)
Fig 6-23. ANOVA analysis of DEXA scan results (g/cm$^3$) at the osteotomy gap three weeks after the operation

Fig 6-24. ANOVA analysis of DEXA scan results (g/cm$^3$) at the osteotomy gap five weeks after the operation

* The number in each bar in Figure 6-23 and 6-24 showed different experimental groups; data points sharing the same Tukey’s letter are not significantly different.
Figure 6-25. A photomicrograph showing bone regeneration in Group 1 (Mag x100)

Figure 6-26. A photomicrograph showing bone regeneration in Group 2 (Mag x100)
Figure 6-27. A photomicrograph showing bone regeneration in Group 3 (Mag x100)

Figure 6-28. A photomicrograph showing bone regeneration in Group 4 (Mag x100)
Figure 6-29. A photomicrograph showing bone regeneration in Group 5 (Mag x100)

Figure 6-30. A photomicrograph showing bone regeneration in Group 6 (Mag x100)
Figure 6-31. Quantitative histological analysis of percentage bone formation at the osteotomy gap

* Data points sharing the same Tukey’s letter are not significantly different.
6.4 DISCUSSION

It has been demonstrated in this study that MSCs enclosed within fibrin glue remained viable up to 96 hours. The viability was 80% of that seen in similar cells grown in optimal monolayer culture. Thus the feasibility of using fibrin glue as a carrier to deliver MSCs is therefore justified. One advantage of fibrin glue is that MSCs can be mixed evenly and into the deep layer. It takes at least a few hours for MSCs to adhere onto hydroxyapatite (HA) in vitro before MSC-HA complex is able to be used and the procedures are more complicated (Bruder 1998a). However, MSCs can be mixed with fibrin glue immediately before use in the operation room and the procedure is simple. A third advantage for fibrin glue is that it is injectable; MSCs can be delivered via injection and this is suitable for filling irregular-shaped defects. Using this method undifferentiated MSCs can be used. For HA carriers, differentiation of MSCs to osteoblasts does occur (Deligiann 2001). There may be advantages of using the undifferentiated MSCs compared with cells differentiated into osteoblasts. Conditions within the site for regeneration may not be ideally suitable for osteoblasts. Chondrocytes prefer lower oxygen tensions and are able to withstand more micromotion than osteoblasts, presumably due to the type of matrix they produce (Hansen 2001). In addition endochondral bone formation is a more common route of
bone regeneration and repair in the adult skeleton than intramembranous bone formation which relies solely on osteoblasts.

It has been demonstrated in my study that MSCs were effective in enhancing bone regeneration in normal and chemotherapy-treated rats. This is an important finding and justifies the use of bone marrow-derived MSCs to facilitate bone regeneration.

Because of the limitation of the sizes of the animals, it is impossible to use autologous MSCs and MSCs from isogenic animals were used instead. It may be worthwhile to repeat the same study on bigger animals such as rabbits or sheep, in which autologous MSCs can be obtained from autologous bone marrow aspiration.

Since the detrimental effects of chemotherapy on MSCs has been clearly demonstrated in the previous chapter (Chapter 3), it is important to take this point into consideration when applying this technology to patients. If autologous MSCs are to be used, it is better to harvest them before the administration of chemotherapy. However, it is extremely difficult to completely rule out the possibility of micrometastasis of the tumour cells into the bone marrow. Allogenic transplantation of MSCs, with or without Human Leukocyte Antigen (HLA) typing, may be a reasonable alternative. There is emerging evidence that MSCs do not express MHC antigens and are
immune-privileged (Klyushnenkova 1998) and use of allogenic MSCs for bone reconstruction has been successfully demonstrated in dogs (Livingston 2001).

In my study, undifferentiated MSCs were used. It is unclear whether the MSCs should be treated with growth factors such as BMP-2 or OP-1 in order to promote the proliferation and differentiation to osteoblastic lineages. It is not clear either as to the optimal concentration and treatment period of each growth factor on MSCs. Further studies are necessary to define whether growth factors are necessary to stimulate the MSCs ex vivo as well as the optimal dosage and treatment timing/duration.

It has been demonstrated in this study that fibrin glue, which is injectable, can be used to deliver MSCs. Fibrin glue has also been used for chondrocyte delivery to reconstruct cartilage defects (Silverman 1999, Peretti 2000). Besides the use in the musculoskeletal system, fibrin glue has been used to deliver cultured autologous urothelial cells for urethral reconstruction (Bach 2001, Wechselburger 1998, Wechselberger 2001). The feasibility of using fibrin glue to deliver anti-cancer chemotherapy drugs has also been investigated (Yoshida 2000).
Therefore, it is concluded from my study that mesenchymal stem cells can survive in fibrin glue up to 96 hour in culture and fibrin glue is suitable for mesenchymal stem cell delivery. I have shown that impaired bone regeneration rate in chemotherapy-treated rats can be accelerated and comparable to normal rats with the help of mesenchymal stem cells, using fibrin glue as a delivery vehicle. The implication of the finding is that, in the reconstruction surgery after the resection of malignant bone tumours using tumour prostheses, it would be useful to apply mesenchymal stem cells using fibrin glue as a delivery vehicle at the bone-implant interface to enhance osseo-integration. Also, for allograft reconstruction, it would also be useful to load mesenchymal stem cells, autologous or allogenic, into the allografts to speed-up new bone formation. The same method can also be applied to autograft reconstruction, such as vascularised fibular graft. MSCs can be applied at the ends of the graft to ensure a rapid healing of the graft bone ends to the host bones.
Chapter Seven

GENERAL DISCUSSION
The aims of the thesis were to quantitatively investigate the detrimental effects of chemotherapy on skeletal cells and tissues as well as fracture healing and to investigate the feasibility of using mesenchymal stem cells to repair bone defects in normal and chemotherapy-treated hosts. The results of the experiments in the previous chapters support the hypotheses that

1. **Chemotherapy drugs, at their therapeutic concentrations, are detrimental to the proliferation and osteogenic differentiation of mesenchymal stem cells.**

2. **Chemotherapeutic agents influence the mechanical properties of the skeletal tissues.**

3. **Chemotherapy treatment slows down the rate of bone regeneration.**

4. **Use of mesenchymal stem cell-based tissue engineering approach increases bone formation at the fracture site.**

In Chapters 4, 5 and 6, the animal model used in the series of studies was the rat. Due to the limitation of the size of the rats, it is impossible to harvest MSCs and transplant them back to the same animal; the rat has to be sacrificed at the time of harvesting the cells. Therefore, isogenic rats have been used for my experiments. However, by definition, allogenic MSCs (although from isogenic animals) have been used. The
results of this study not only encourage the use of autologous MSCs but also have thrown light on the use of allogeneic MSCs. In a study using marrow-derived progenitor cells to enhance new bone formation during distraction osteogenesis (Richards 1999), isogenic Sprague-Dawley rats were used. Although cell syngenicity assay showed that the donor and recipient rats were not completely compatible, the rats that received cells had significantly more new bone formation at the distraction site. Recently, it has been demonstrated that allogeneic MSCs can successfully repair segmental bone defects along with hydroxyapatite scaffolds in a canine model (Livingston 2001). Also, there is in vitro evidence that MSCs do not elicit an immune response when combined with allogeneic lymphocytic cells in immunologic studies (Klyushnenkova 1998).

The other argument about using a rat model for bone regeneration study is that rats have greater potential of growing bones than other animals. Therefore, it would be better to use animals of larger size such as rabbits or dogs. However, due to the strict regulation of the Home Office, it is more difficult to obtain a project license to work on dogs than rabbits or rats. This, when combined with cost implications, means that the rat is a suitable animal to use for proving concepts which may later be applied to the human scenario. The problem of using autologous rabbit MSCs is that the
variability of bone marrow-derived MSCs is high in rabbits (Solchaga 1999) and their osteogenic potential is influenced by the age and sampling site of the rabbits (Huibregtse 2000). Therefore, rats were used in the experiments.

Any new concept for bone regeneration needs to be proved in animals. Remodelling of bones in rats is faster than in humans and in many other animals. Therefore, the fact that this concept of MSC augmentation is advantageous in rats does not necessarily apply to humans. That is the reason why a number of experiments involving bone remodelling have been carried out in larger species such as dogs and sheep. It would be even more convincing if permission to work on dogs or sheep could be obtained and the same experiments are repeated on dogs in the future.

Mechanical factors play an important role in animal models for bone regeneration (Lanyon 1982; Carter 1998). In the majority of the animal experiments involving the use of MSCs to enhance bone regeneration, internal fixators were used (Kadiyala 1997; Bruder 1998a; Bruder 1998b). In fact, the micro-motion around the fracture gap, as well as the implant stiffness and the pre-load of the implants, all influence the outcome of bone regeneration (Terjesen and Apalset 1988; Kenwright 1991; Goodship 1993; Goodship 1998). The disadvantage of using internal fixation is that
the mechanical environment is not as well controlled as external fixators. Therefore it can cause bias in the results. On the other hand, with external fixators, the mechanical loading was more consistent. That is the reason why external skeletal fixtators were used in Chapter 5 and Chapter 6. This is also important for MSCs because these cells also respond differently to different mechanical environments (Carter 1998).

In Chapter 4, systemic effects of cisplatin and doxorubicin on skeletal tissues have been shown. It would be interesting to know whether reduced mechanical properties of the skeletal tissues can be reversed by pharmacological approaches, particularly calcitonin and bisphosphonates (Li 1996). It would also be interesting to find out whether MSCs can help reverse the detrimental effects by transplanting large quantities of MSCs systemically through infusion (Lazarus 1995, Koc 2000).

In Chapter 5, detrimental effects of cisplatin and doxorubicin on fracture healing have been shown. This implies that when reconstructing bone defects in limb salvage procedures that involve the use of segmental autograft or allograft, healing of the graft-host bone junction may be slowed by chemotherapy. Again, it is worthwhile investigating the feasibility of using MSCs to accelerate the healing of graft-host bone junctions in chemotherapy-treated animals in the future.
In Chapters 3 and 4, the toxicity of cisplatin and doxorubicin on MSCs and skeletal tissues was clearly demonstrated in my study. Although this two-drug regime is more tolerable by the patients (Souhami 1997), it is not without problems. Actually, according to the results of my study, chemotherapy influences skeletal tissues significantly. Since this two-drug regime has become the gold standard chemotherapy protocol for malignant bone tumours in the United Kingdom as well as many other European countries, it is important for surgeons and physicians to bear in mind that cisplatin and doxorubicin affect skeletal tissues. The implications of this fact are extensive; during surgical resection of the tumour and reconstruction with prosthetic implant, the chance of intra-operative fracture can be higher because the bones are weakened. Also, after the operation, the chance of peri-prosthetic fracture may also be increased for the same reason. Segmental reconstruction after tumour resection could be one of the applications of this therapy and as these patients undergo a regime of chemotherapy, it is important to discover if a tissue engineering approach is warranted on these patients who are compromised. The time for hydroxyapatite-coated massive prostheses to achieve osseo-integration may be increased compared with these coatings in more conventional hip replacements because the abilities of proliferation and osteogenic differentiation of MSCs are affected by cisplatin and doxorubicin.

From this viewpoint, it is therefore useful to apply a tissue engineering approach to
CHAPTER 7

enhance osseo-integration around the implant when the bone regeneration power of
the patient is compromised. This technique may not only be useful to aid the
integration of bone tumour implants but could also be used, for example, in patients
with osteoporosis and patients of advanced age who need hip or knee replacements.
Human leukocytic antigen (HLA)-matched, allogenic mesenchymal stem cell
transplantation from young, healthy donors may be a solution.

The other possible approach is to apply growth factors such as BMP-2 or OP-1 at the
bone-implant interface. A third approach, which combines tissue engineering with
gene therapy, may also be an alternative. Genetically-modified autologous MSCs that
carry genes coding for BMP-2 or OP-1 (Caplan 2000; Mosca 2000; Turgeman 2001)
can be used to enhance osseo-integration. The advantage of using MSCs with genes
coding for growth factor is that the half-life of growth factor is short and the
expression of growth factor in these genetically-modified MSCs can be much longer
(Nevo 1998; Gazit 1999; Baltzer 2000; Kaji and Leiden 2001). There is in vitro
evidence that human mesenchymal stem cells maintain transgene expression during
expansion and differentiation (Allay 1997; Lee 2001). Besides, the expression of
BMP-2 gene can be controlled by doxycycline if a tetracycline-regulated expression
vector encoding BMP-2 is harbored in MSCs (Moutsatsos 2001). With this “switch”
mechanism, the timing as well as the level of expression of growth factor can be controlled and thus the growth factor can be “turned on” and “turned off” at the desired time points. The same approach can be used for fracture healing and the regeneration of bone defects (Niyibizi 1998; Niyibizi and Kim 2000) as well as treatment of osteoporosis (Kale and Long 2000). Genetic skeletal disease, in particular, osteogenesis imperfecta (OI), is a potential candidate to be treated with MSCs (Caplan 1995; Pereira 1998) or gene therapy using MSCs as a carrier (Niyibizi 2000).

This is a dominant negative disorder of connective tissue. Defects in COL1A1 or COL1A2 genes, coding for the α1 and α2 chains of type 1 collagen respectively, are the causative mutations. Over 150 mutations have been characterized. Both quantitative defects, such as null COL1A1 alleles, and qualitative defects, such as glycine substitution, exon skipping, deletions, and insertions, have been described (Forlino 2000). Transplantation of HLA-matched MSCs after chemotherapy has been undergoing clinical trial and the early results are encouraging (Horwitz 1999; Horwitz 2001). Another approach by transferring proα2(I) cDNA to MSCs and then transplanting the cells to the animals in an osteogenesis imperfecta mice (oim) model, has shown successful correction of the type 1 collagen production (Niyibizi 2001).

Pre-natal transplantation of stem cells is an exciting frontier for the treatment of many
congenital diseases. The fetus may be an ideal recipient for stem cells as it is
immunologically immature and has rapidly proliferating cellular compartments that
may support the engraftment of transplanted cells. MSCs, given their ability of
differentiating into multiple cell types, could potentially be used to treat diseases such
as OI, muscular dystrophy and other mesenchymal disorders that can be diagnosed in
utero. Very recently, it has been shown that human mesenchymal stem cells persist
and demonstrate site-specific multipotential differentiation and are present in sites of
wound healing and tissue regeneration after transplantation into fetal sheep (Liechty
2000; Mackenzie and Flake 2001). These findings support the use of MSCs for
intrauterine therapy.

Most of the researchers on bone regeneration use bone marrow-derived MSCs. Recent
studies show that besides bone marrow, muscles (Lee 2000; Musgrave 2000) and
adipose tissues (Halvorsen 2000; Zuk 2001), with or without genetic modification,
can also provides stem cells for bone regeneration.

It was previously reported that MSCs were not able to be collected from the
peripheral blood in human (Lazarus 1997). It was, however, subsequently found by
other researchers that mesenchymal precursor cells could be obtained from peripheral
blood of normal individuals (Zvaifer 2000). In that study, mesenchymal precursor
cells harvested from peripheral blood expressed CD-105 (endoglin), a putative marker
for bone-marrow MSCs. Also, those cells responded to osteogenic supplements and
BMP-2, increasing the expression of alkaline phosphatase and 30% of the cells
expressed osteocalcin after two weeks. The same culture also contained sudanophilic
adipocytes. The most striking breakthrough is that fibroblast-like cells with
osteogenic and adipogenic potential have been successfully isolated from the
circulating peripheral blood of four mammalian species including human (Kuznetsov
2001). In that study, those fibroblast-like cells phenotypically resemble, but are
distinguishable from, MSCs. This is the first definitive proof of the existence of
circulating skeletal stem cells in mammals. Peripheral blood-derived skeletal stem
cells may have a lot of advantages over bone marrow-derived MSCs. Peripheral blood
is obviously more accessible compared with bone marrow; the procedure is
straightforward, no anaesthesia is needed and the discomfort to the patient is
minimized.

The most striking recent finding in terms of sources of stem cells is that a novel cell
type, which is spore-like, seems to be present in all tissues in adult mammals (Vacanti
2001). According to the authors of that study, these spore-like cells, less than 5 µm in
size, appear to lie dormant and are dispersed throughout the parenchyma of virtually
every tissue. However, these cells have an exceptional ability to survive in hostile
conditions and it is believed that these unique cells lie dormant until activated by
injury or disease, when they have the potential to regenerate tissues lost to disease or
damage.

In this study, fibrin glue was used to deliver MSCs for bone regeneration. It has been
demonstrated both in vitro and in vivo that fibrin glue is suitable for MSC delivery.

Because of the injectability of fibrin glue, it is especially useful in delivering MSCs to
contained, irregular bone defects. There are a number of clinical applications. For
example, after curettage of benign bone tumours such as simple bone cyst, aneurismal
bone cyst and giant cell tumour, fibrin glue containing MSCs can be injected to the
defects. Currently, polymethymethacrylate (PMMA) bone cement, bone grafts or bone
graft substitutes such as hydroxyapatite, tri-calcium phosphate and calcium sulphate
are being used. Fibrin glue containing MSCs may be a better adhesive. Another
extremely useful indication is in joint replacement surgery. For example, in patients
with silent osteolysis around the acetabular component years after primary total hip
replacement, MSCs could be delivered to the defects along with fibrin glue via a
minimally invasive approach. The same method can be used for filling in bone defects
around the cementless hip stems which are still stable where the loosening process is still in the initial stages. Suitable candidates for this type of therapy may also include patients with osteolytic lesions around the total knee prostheses caused by wear debris.

It may also be possible to apply MSC-containing fibrin glue at the bone-implant interface of cementless hip and knee implants in order to achieve faster osseo-integration. A third useful application is in spine surgery. For example, compression fractures of the spine caused by osteoporosis are extremely difficult to treat. Currently, surgeons inject PMMA or calcium phosphate bone cement percutaneously into the vertebral bodies of the patients with osteoporotic compression fractures (Cyteval 1999; Barr 2000; Heini 2000). MSC-containing fibrin glue may also well be an option for percutaneous vertebroplasty.

For tissue-engineered bone regeneration, hydroxyapatite (HA) is the most extensively studied and used material because HA itself is osteoconductive. It is true that HA has much stronger mechanical strength than fibrin glue, but it is not without problems. MSCs can be mixed into fibrin glue which will set immediately right before use. It will take much longer for MSCs to adhere onto HA blocks. Besides, MSCs can also be mixed thoroughly and distributed evenly in fibrin glue; however, it would be difficult for MSCs to be delivered or migrate into the inner part of HA blocks. To
solve these problems regarding the loading and migration of MSCs on HA scaffolds, as well as the delivery of nutrients, various bioreactors were designed (Porter 2001). Bioreactors not only allow better perfusion of the culture media, they also provide a three-dimensional culture environment (Granet 1998) as three-dimensional cellular development is essential for ex vivo formation of human bone (Kale 2000). It has been shown that cell growth and differentiation is to be influenced by bioreactor geometry (Peng and Palsson 1996). Also, in some designs of the bioreactors, rotating-wall vessels are used (Qiu 1998, Qiu 1999, Botchway 2001), which implied that constant mechanical stimulus is applied. This is important because bone progenitor cells do respond to mechanical stimulus. Further studies are encouraged to define the optimal mechanical stimulus for MSCs to proliferate and differentiate into different lineages.

The progress of tissue engineering is always amazing and surprising. Organ shortage and suboptimal prosthetic or biologic materials for repair or replacement of diseased or destroyed human organs and tissues are the main motivation for increasing research in the emerging field of tissue engineering (Stock and Vacanti 2001).

It is foreseeable that in the near future, treatment of many diseases will change
considerably because of the advances of tissue engineering, particularly in the field of orthopaedics. To achieve this goal, continuous efforts in the research and development of new technologies in orthopaedic tissue engineering is a must. Besides, the collaboration among clinicians and scientists in different disciplines are of crucial importance in order to apply the new inventions from bench to bedside as quickly as possible. Most important of all, no matter how far the technologies in tissue engineering advances, it always has to be kept in mind that the ultimate goal of medical professionals is to help mankind and respect life, which is invaluable. Whoever has the knowledge and technique of tissue regeneration should not abuse it and should not use it for the purposes other than the welfare of those who need medical help.
REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


Caplan AI: Osteogenesis imperfecta, rehabilitation medicine, fundamental research and mesenchymal stem cells. Conn Tissue Res 31: S9-S14 (1995)

REFERENCES


REFERENCES


Chesney J, Bacher M, Bender A, Bucala A. The peripheral blood fibrocyte is a potent antigen-presenting cell capable of priming naïve T cells in situ. Proc Natl Acad Sci USA 94: 6307-6312 (1997)


Debiais F, Hott M, Graulet AM, Marie PJ. The effects of fibroblast growth factor-2 on human neonatal calvaria osteoblastic cells are differentiation specific. J Bone
REFERENCES


REFERENCES

Digioholm CM, Stokes D, Colter D, Phinney DG, Class R and Prockop DJ.


Donnelly WJ, Kobayashi A, Freeman MA, Chin TW, Yeo H, West M, Scott G.


REFERENCES


REFERENCES


REFERENCES


REFERENCES


Glackin CA, Murray EJ, Murray SS. Doxorubicin inhibits differentiation and enhances expression of the helix-loop-helix genes Id and mTwi in mouse osteoblastic cells. Biochem Int 28: 67-75 (1992)
REFERENCES


Grzesiak JJ, Pierschbacher MD, Amodeo MF, Malaney TI, Glass JR.

Guest I, Uetrecht J. Drugs toxic to the bone marrow that target the stromal cells. Immunopharmacology 46: 103-112 (2000)


REFERENCES

Hansen U, Schunke M, Domm C, Ioannidis N, Hassenpflug J, Gehrke T, Kurz B.
Combination of reduced oxygen tension and intermittent hydrostatic pressure: a useful tool in articular cartilage tissue engineering. J Biomech 34: 941-949 (2001)


REFERENCES

Haynesworth SE, Baber MA, Caplan AI. Cell surface antigens on human
marrow-derived mesenchymal cells are detected by monoclonal antibodies. Bone 13:
69-80, (1992a)

Haynesworth SE, Goshima J, Goldberg VM, Caplan AI. Characterization of cells
with osteogenic potential from human bone marrow. Bone 13: 81-88 (1992b)

Haynesworth SE, Baber MA, Caplan AI. Cytokine expression by human
marrow-derived mesenchymal progenitor cells in vitro: expression effects of

Haynesworth SE, Reuben D, Caplan AI. Cell-based tissue engineering therapies:

Heini PF, Walchli B, Berlemann U. Percutaneous transpedicular vertebroplasty with
PMMA: operative technique and early results. A prospective study for the treatment

Hejna MJ, Gitelis S. Allograft prosthetic composite replacement for bone tumors.
REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


Johnson EE, Urist MR, Finerman GAM: Resistant nonunions and partial or complete segmental defects of long bones: Treatment with implants of a composite of human bone morphogenetic protein (BMP) and autolyzed, antigen-extracted, allogenic (AAA) bone. Clin Orthop 277: 229-237 (1992)


REFERENCES


REFERENCES


REFERENCES


REFERENCES


Klyushnenkova E, Mosca JD, McIntosh KR. Human mesenchymal stem cells suppress allogenic T cell response in vitro: Implications for allogenic transplantation. Blood 82 suppl 1: 642a (1998)


REFERENCES


REFERENCES


Lazarus HM, Haynesworth SE, Gerson SL, Caplan AI. Human bone marrow-derived mesenchymal (stromal) progenitor cells (MPCs) cannot be recovered from peripheral blood progenitor cell collections. J Hematother 6: 447-455 (1997)


REFERENCES


REFERENCES


Livingston T, Kaliyala S, ElKalay M, Young R, Kraus K, Gordon S, Peter S. Repair of canine segmental bone defects using allogenic mesenchymal stem cells. Trans ORS 26: 49 (2001)

REFERENCES


Lyons KM, Hogan BL, Robertson EJ. Colocalization of BMP 7 and BMP 2 RNAs suggests that these factors cooperatively mediate tissue interactions during murine development. Mech Dev 50: 71-83 (1995a)


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


Peng CA, Palsson BO. Cell growth and differentiation on feeder layers is predicted to be influenced by bioreactor geometry. Biotech Bioeng 50: 479-492 (1996)


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


Scutt A, Bertram P. Basic fibroblast growth factor in the presence of dexamethasone stimulates colony formation, expansion, and osteoblastic differentiation by rat bone marrow stromal cells. Calcif Tissue Int 64: 69-77 (1999)


REFERENCES


REFERENCES


REFERENCES


REFERENCES


Urist MR. Practical application of basic research on bone graft physiology. AAOS Instr Course Lec 26: 1-26 (1976)


Uyttendaele D, De Schryver A, Claessens H, Roels H, Berkvens P, Mondelaers W.


Van den Bos C, Mosca JD, Winkles J, Kerrigan L, Burgess WH, Marshak DR.

Human mesenchymal stem cells respond to fibroblast growth factors. Hum Cells 10: 43-50 (1997)


REFERENCES


REFERENCES


REFERENCES

Wheeler DL, Vander Griend RA, Wronski TJ, Miller GJ, Keith EE, Graves JE.

Wheeler DL, Graves JE, Miller GJ, Vander Griend RE, Wronski TJ, Powers SK,
Park HM. Effects of running on the torsional strength, morphometry, and bone mass

Whitby DJ, Ferguson MWJ. The extracellular matrix of lip wounds in fetal,

Wikesjo UME, Sorensen RG, Wozney JM. Augmentation of alveolar bone and
dental implant osseointegration: clinical implications of studies with rhBMP-2. J

Biocompatibility analysis of different biomaterials in human bone marrow cell
REFERENCES


REFERENCES


Appendix I: Methods used in the study:

(1) $^3$H-THYMIDINE INCORPORATION USING THE MULTISCREEN SYSTEM

REFERENCES

Maurer HR (1981): Potential pitfalls of $^3$H-thymidine techniques to measure cell proliferation. *Cell Tissue Kinet* 14 111-120


PRINCIPLE

Radiolabelled thymidine is added to cell for a given period of time and in incorporated into newly-formed DNA. The cells are harvested and DNA is precipitated with trichloroacetic acid; the precipitate is collected on membranes at the bottom of the wells of a 96well filtration plate. These are punched into scintillation vials for counting and cell growth and proliferation can be correlated.

REAGENTS

1. TRICHLOROACETIC ACID (TCA) 10%

   For 500ml, weigh 50g TCA (MERCK) into a 500ml beaker and add 450ml distilled water. Mix thoroughly until dissolved then transfer to a 500 ml volumetric flask and make up to volume with distilled water. Store at 4°C.

2. TRICHLOROACETIC ACID (TCA) 20%

   Make up as for 10% solution but weigh out 100g. Store at 4°C.

3. POTASSIUM HYDROXIDE 0.01M

   For 500ml, weigh 0.281 g KOH pellets into a 500ml beaker and add 450ml distilled water. Mix thoroughly until dissolved the transfer to a 500ml volumetric flask and make up to volume with distilled water.

4. SCINTILLATION COCKTAIL

   The scintillant in current use in Packard Scintillator Plus (Canberra Packard).

METHOD:

1. Add 200μl 20% TCA (at 4°C) to al wells of a Millipore filter plate (65μm pore
APPENDIX I

If any wells are left dry, the vacuum will be incomplete.

2. Aspirate 20% TCA using Multiscreen manifold.

3. Draw up a plate map as shown overleaf.

4. Add 100μl samples to the appropriate wells, followed by 100μl 20% TCA (at 4°C).

5. Incubate at 4°C for 30 minutes, with gentle agitation than aspirate as before.

6. Rinse wells twice with 200μl 10% TCA, aspirating each time.

7. Remove plastic underside of plate. Blot filters and leave to dry at 37°C for at least 45 minutes.

8. Punch filter membranes into scintillation vials.

9. Add 500μl KOH (0.01M) to vials, transfer vials to counter racks and mix on Luckham mixer for hours (alternatively, samples can be left at 4°C overnight).

10. Add 4ml scintillant, cap and vortex. Count on β-counter.

11. PLATE MAP FOR 3H-THYMIDINE INCORPORATION

<p>| | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>G</td>
<td>F</td>
<td>E</td>
<td>D</td>
<td>C</td>
<td>B</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>U1</td>
<td>U1</td>
<td>U2</td>
<td>U2</td>
<td>U3</td>
<td>U3</td>
<td>U4</td>
<td>U4</td>
<td>1</td>
</tr>
<tr>
<td>U5</td>
<td>U5</td>
<td>U6</td>
<td>U6</td>
<td>U7</td>
<td>U7</td>
<td>U8</td>
<td>U8</td>
<td>2</td>
</tr>
<tr>
<td>U9</td>
<td>U9</td>
<td>U10</td>
<td>U10</td>
<td>U11</td>
<td>U11</td>
<td>U12</td>
<td>U12</td>
<td>3</td>
</tr>
<tr>
<td>U13</td>
<td>U13</td>
<td>U14</td>
<td>U14</td>
<td>U15</td>
<td>U15</td>
<td>U16</td>
<td>U16</td>
<td>4</td>
</tr>
<tr>
<td>U17</td>
<td>U17</td>
<td>U18</td>
<td>U18</td>
<td>U19</td>
<td>U19</td>
<td>U20</td>
<td>U20</td>
<td>5</td>
</tr>
<tr>
<td>U21</td>
<td>U21</td>
<td>U22</td>
<td>U22</td>
<td>U23</td>
<td>U23</td>
<td>U24</td>
<td>U24</td>
<td>6</td>
</tr>
<tr>
<td>U25</td>
<td>U25</td>
<td>U26</td>
<td>U26</td>
<td>U27</td>
<td>U27</td>
<td>U28</td>
<td>U28</td>
<td>7</td>
</tr>
<tr>
<td>U29</td>
<td>U29</td>
<td>U30</td>
<td>U30</td>
<td>U31</td>
<td>U31</td>
<td>U32</td>
<td>U32</td>
<td>8</td>
</tr>
<tr>
<td>U33</td>
<td>U33</td>
<td>U34</td>
<td>U34</td>
<td>U35</td>
<td>U35</td>
<td>U36</td>
<td>U36</td>
<td>9</td>
</tr>
<tr>
<td>U37</td>
<td>U37</td>
<td>U38</td>
<td>U38</td>
<td>U39</td>
<td>U39</td>
<td>U40</td>
<td>U40</td>
<td>10</td>
</tr>
<tr>
<td>U41</td>
<td>U41</td>
<td>U42</td>
<td>U42</td>
<td>U43</td>
<td>U43</td>
<td>U44</td>
<td>U44</td>
<td>11</td>
</tr>
<tr>
<td>U45</td>
<td>U45</td>
<td>U46</td>
<td>U46</td>
<td>U47</td>
<td>U47</td>
<td>U48</td>
<td>U48</td>
<td>12</td>
</tr>
</tbody>
</table>
(2) FLUORIMETRIC ASSAY OF DNA USING HOECHST 33258

REFERENCES


PRINCIPLE OF THE ASSAY

The fluorimetric sye, Hoechst 33258, is DNA-specific and binds to contiguous adenine-thymine base pairs emitting fluorescence at a wavelength of 460nm.

INSTRUMENTATION

Ascent fluorimetric plate reader (see separate SOP for its use)
Balance (4 decimal place)
P1000, P200, P20 automatic pipettes
Multichannel pipette (8 or 12 channel)
pH meter

EQUIPMENT

Blue and yellow tips
Universals
Graduated pipettes
White Fluoronunc plates
Plate sealers

HAZARD WARNING

THE HOECHST REAGENT BINDS TO DNA AND IS MUTAGENIC-GLOVES MUST BE WORN WHEN CARRYING OUT THIS ASSAY.

REAGENTS

1. SALINE SODIUM CITRATE (SSC) buffer, pH 7.0.

   To prepare 500ml of stock (20x working concentration) (which must be diluted 1 in 20 to give working reagent for use in assay).

   Sodium chloride 87.65g
   Trisodium citrate 44.1g
APPENDIX I

Make up to 480ml with laboratory grade water (from Elga in Room 46) and adjust pH to 7.0 with 1M sodium hydroxide or 1M hydrochloric acid if necessary. Make up to full volume, aliquot if required and sterilise by autoclaving.

2. HOECHST 33258 (SIGMA B2883)-Aliquots of a stock solution of 1mg/ml in distilled deionised water are to be used.

3. DNA STANDARD (from calf thymus SIGMA D3664)-Aliquots of a stock standard of 1mg/ml in water are to be used.

NOTES

- It may be necessary to modify the buffer system used in the assay according to the buffer system used in preparation of your samples; check with your supervisor for clarification.

- The volumes of sample/standard and the concentration and volume of the Hoeschst reagent can be modified slightly to alter the sensitivity of the assay if your samples require it.

- The assay is sensitive down to 0.125μg/ml using this method.

- It is possible to use your own modified plate layout if you wish and store this on the PC attached to the Ascent reader.

METHOD

1. Prepare plate maps assigning standards and samples as shown below:

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>U1</td>
<td>U2</td>
<td>U3</td>
<td>U4</td>
<td>U5</td>
<td>U6</td>
<td>U7</td>
<td>U8</td>
<td>U9</td>
</tr>
<tr>
<td>B</td>
<td>0.31</td>
<td>0.31</td>
<td>0.31</td>
<td>U</td>
<td>U2</td>
<td>U3</td>
<td>U4</td>
<td>U5</td>
<td>U6</td>
<td>U7</td>
<td>U8</td>
<td>U9</td>
</tr>
<tr>
<td>C</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
<td>U1</td>
<td>U11</td>
<td>U12</td>
<td>U13</td>
<td>U14</td>
<td>U15</td>
<td>U16</td>
<td>U17</td>
<td>U18</td>
</tr>
<tr>
<td>D</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>U10</td>
<td>U11</td>
<td>U12</td>
<td>U13</td>
<td>U14</td>
<td>U15</td>
<td>U16</td>
<td>U17</td>
<td>U18</td>
</tr>
<tr>
<td>E</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>U10</td>
<td>U20</td>
<td>U21</td>
<td>U22</td>
<td>U23</td>
<td>U24</td>
<td>U25</td>
<td>U26</td>
<td>U27</td>
</tr>
<tr>
<td>F</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>U19</td>
<td>U20</td>
<td>U21</td>
<td>U22</td>
<td>U23</td>
<td>U24</td>
<td>U25</td>
<td>U26</td>
<td>U27</td>
</tr>
<tr>
<td>G</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>U19</td>
<td>U29</td>
<td>U30</td>
<td>U31</td>
<td>U32</td>
<td>U33</td>
<td>U34</td>
<td>U35</td>
<td>U36</td>
</tr>
<tr>
<td>H</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
<td>U28</td>
<td>U29</td>
<td>U30</td>
<td>U31</td>
<td>U32</td>
<td>U33</td>
<td>U34</td>
<td>U35</td>
<td>U36</td>
</tr>
</tbody>
</table>
APPENDIX I

Number plates and plate maps accordingly.

2. PREPQRTION OF STANDARD CURVE

Work out the total volume of each standard required for the number of plates being run (each plate requires 0.5ml of each standard so, for example 3 plates will require 1.5ml of each standard). Label a series of tubes with the standard concentrations (20, 10, 5, 2.5, 1.25, 0.625 and 0.3125μg/ml).

Prepare 1ml 20μg/ml standard for each plate to be run (eg prepare 3ml if you are running 3 plates) by diluting the stock standard q in t0 in working strength SSC (replace 20μl SSC from each ml with 20μl stock standard) and mixing thoroughly.

Measure a volume of working SSC equivalent to the final volume of standard required (eg for 3 plates 1.5ml SSC) into each of the remaining tubes. Transfer an equal volume of the 20 μg/ml standard to the 10 μg/ml tube and mix well. Repeat, transferring 10 μg/ml standard into the 5μg/ml tube and so on until serial dilutions sown to 0.3125 μg/ml are prepared, mixing thoroughly after each addition.

3. Using working SSC as the 0μg/ml standard, pipette 100μl each standard or sample into wells as appropriate.

4. Work out the total volume of Hoechst reagent required for the assay (a full 96-well plate will require 10ml reagent) and measure that volume of working strength SSC into a universal. Remove the volume of SSC equivalent to the volume of Hoechst 33258 to be added (eg if preparing 10 ml reagent, remove 10μl SSC) and replace it with stock Hoechst 33258. This reagent can be stored for up to 2 hours without loss of activity if it is necessary to repeat the assay.

5. Add 100μl Hoechst 33258 (1μg/ml) to each well using the multichannel pipette. Cover the plate with a plate sealer.

6. Remove the plate sealer and read plate on fluorimeter selecting the protocol dna.prt and using the plate layout cedna.plt

7. Rinse the plate out (in radioactive sink if necessary) thoroughly and place it in a solid waste bin if radioactivity has been used.
(3) ALKALINE PHOSPHATASE ASSAY-MICROPLATE METHOD

REFERENCES


PRINCIPLE

The enzyme alkaline phosphatase cleaves the phosphate group from p-nitrophenyl phosphate to produce p-nitrophenol which is yellow at alkaline pH and can be monitored at or around 405nm. THIS METHOD DOES NOT MEASURE THE AMOUNT OF ENZYME PROTEIN PRESENT DIRECTLY.

RAGENT

1. PBS

2. GLYCINE 0.1M pH 10.3

   To prepare 100, weigh 0.751g glycine powder into a beaker and make up to 90ml with distilled water. Adjust pH to 10.3 as necessary, transfer to a 100ml volumetric flask and make up to final volume.

3. SUBSTRATE REAGENT

   This should be prepared fresh for each run. To make up 10ml (enough for 2 plates), weigh into a universal:

   p-Nitrophenol phosphate 40mg (1 tab)

   Magnesium chloride hexahydrate 34mg

   Triton X-100(12.5% v/v) 80μl

   Add 10ml 0.1M Glycine, pH 10.3 and mix thoroughly.

4. p-NITROPHENOL STANDARD 200Mg/ml

   This should be prepared fresh for each run by weighing 20mg p-nitrophenol (sigma 102-8) into a 100 ml volumetric flask and making up to volume with 0.1M glycine, pH 10.3.
APPENDIX I

METHOD

1. If the assay is being carried out on cell in culture, remove medium and wash well with 100µl PBS; remove this and add 50 µl distilled water to al wells. Place plates at -70°C for 20 minutes then transfer to 37°C room for 15 minutes.

Repeat this twice more to lyse the cell membranes.

If the assay is being carried out on aqueous samples eg medium, cell lysates/digests, transfer 50µl sample to appropriate wells(see plate map below).

2. Prepare serial dilutions of p-nitrophenol standard down to 3.125µg/ml and pipette 100µl of each standard into appropriate wells (see plate map below).

3. Add 50µl substrate solution to all sample wells, cover the plate and shake it for 2 minutes on the Titertek plate shaker.

4. Read absorbance immediately at 410nm (reference wavelength 630nm) on the Dynatech reader or 405nm on the Biorad reader and again at 1,5,10 anf 20 minutes.

PLATE MAP FOR ALP ASSAY

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>U1</td>
<td>U2</td>
<td>U3</td>
<td>U4</td>
<td>U5</td>
<td>U6</td>
<td>U7</td>
<td>U8</td>
<td>U9</td>
</tr>
<tr>
<td>B</td>
<td>3.13</td>
<td>3.13</td>
<td>3.13</td>
<td>U1</td>
<td>U2</td>
<td>U3</td>
<td>U4</td>
<td>U5</td>
<td>U6</td>
<td>U7</td>
<td>U8</td>
<td>U9</td>
</tr>
<tr>
<td>C</td>
<td>6.25</td>
<td>6.25</td>
<td>6.25</td>
<td>U10</td>
<td>U11</td>
<td>U12</td>
<td>U13</td>
<td>U14</td>
<td>U15</td>
<td>U16</td>
<td>U17</td>
<td>U18</td>
</tr>
<tr>
<td>D</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>U10</td>
<td>U11</td>
<td>U12</td>
<td>U13</td>
<td>U14</td>
<td>U15</td>
<td>U16</td>
<td>U17</td>
<td>U18</td>
</tr>
<tr>
<td>F</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>U19</td>
<td>U20</td>
<td>U21</td>
<td>U22</td>
<td>U23</td>
<td>U24</td>
<td>U25</td>
<td>U26</td>
<td>U27</td>
</tr>
<tr>
<td>G</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>U28</td>
<td>U29</td>
<td>U30</td>
<td>U31</td>
<td>U32</td>
<td>U33</td>
<td>U34</td>
<td>U35</td>
<td>U36</td>
</tr>
<tr>
<td>H</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>U28</td>
<td>U29</td>
<td>U30</td>
<td>U31</td>
<td>U32</td>
<td>U33</td>
<td>U34</td>
<td>U35</td>
<td>U36</td>
</tr>
</tbody>
</table>
(4) ALKALINE PHOSPHATASE ASSAY ON COBAS BIO

REFERENCES


PRINCIPLE

The enzyme alkaline phosphatase cleaves the phosphate group from p-nitrophenyl phosphate to produce p-nitrophenol which is yellow at alkaline pH and can be monitored at or around 405nm. The analyser calculates the results using the reaction rate method i.e. a series of absorbance readings is taken and plotted against time to give a slope which indicates the rate of appearance of the product colour; the steeper the slope the greater the enzyme activity. THIS METHOD DOES NOT MEASURE THE AMOUNT OF ENZYME PROTEIN PRESENT DIRECTLY.

REAGENTS

1. P-NITROPHENOL PHOSPHATE POWDER

   This is supplied as a pre-weighed powder (RANDOX)

2. DIETHANOLAMINE BUFFER

   This is supplied ready to use (RANDOX)

METHOD

1. Remove samples and controls from the freezer on the day before the assay is to be run and leave to thaw at 4°C overnight. This allows for complete regeneration of the enzyme activity.

2. Pipette 50μl samples, controls into the blue sample cups designed for the Cobas Bio (see Caroline or Kanta for these.)

3. Prepare working reagent by adding 8ml buffer to 1 bottle of substrate powder. The assay requires 0.25ml reagent per test plus 0.3ml to allow for dead space in the reagent boat. The reagent is stable for 30 days at 4°C and 3 days at room temperature.

4. Take samples, controls and reagents to the metabolic Lab to run the Assay.
Transfer the substrate reagent to the boat as shown overleaf and lead the sample cups into the sample rotor.

5. Select 'Randox ALP' (key no 6) on the Cobas Bio and run assay following the protocol for using the Cobas in the equipment handbook.

**COBAS BIO PARAMETERS FOR ALP ASSAY**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculation factor</td>
<td>2698</td>
</tr>
<tr>
<td>Standard 1</td>
<td>0</td>
</tr>
<tr>
<td>Standard 2</td>
<td>0</td>
</tr>
<tr>
<td>Standard 3</td>
<td>0</td>
</tr>
<tr>
<td>Limit</td>
<td>0.65</td>
</tr>
<tr>
<td>Temperature°C</td>
<td>37.0</td>
</tr>
<tr>
<td>Type of analysis</td>
<td>2</td>
</tr>
<tr>
<td>Wavelength nm</td>
<td>405</td>
</tr>
<tr>
<td>Sample volume (μl)</td>
<td>0.05</td>
</tr>
<tr>
<td>Diluent volume (μl)</td>
<td>0.55</td>
</tr>
<tr>
<td>Reagent (oxidant) volume (μl)</td>
<td>2.50</td>
</tr>
<tr>
<td>Incubation time (s)</td>
<td>0</td>
</tr>
<tr>
<td>Start reagent volume (μl)</td>
<td>0</td>
</tr>
<tr>
<td>Time of first reading (s)</td>
<td>50</td>
</tr>
<tr>
<td>Time intervals (s)</td>
<td>10</td>
</tr>
<tr>
<td>Number of readings</td>
<td>15</td>
</tr>
<tr>
<td>Blanking mode</td>
<td>1</td>
</tr>
<tr>
<td>Printout mode</td>
<td>1</td>
</tr>
</tbody>
</table>
APPENDIX I

(5) MEASUREMENT OF CELL PROLIFERATION USING ALAMAR BLUE

REFERENCES


PRINCIPLE

Alamar Blue is a REDOX indicator i.e., it responds to reduction or oxidation of the surrounding medium. In this assay it both fluoresces and changes colour in response to the chemical reduction of culture medium which results from cell growth and division. The Alamar Blue can then be removed and replaced with fresh medium so that monitoring can be continued.

REAGENTS

1. COMPLETE CULTURE MEDIUM-PHENOL-RED FREE

2. ALAMAR BLUE SOLUTION

This is purchased as a 10x concentrate from Serotec(cat no. BUF012A for 25ml). It is diluted 1 in 10 with pr-free medium.

3. PHOSPHATE-BUFFERED SALINE(PBS)

METHOD

NOTE: The assay should be carried out on cells that have reached the log phase of their growth cycle.

1. Remove the medium from all the wells under test.

2. Dilute 10x Alamar Blue with PR-free medium to give enough solution for your
requirements.

3. Add the appropriate volume for your application to each well.

<table>
<thead>
<tr>
<th>APPLICATION</th>
<th>VOLUME ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell treated with GFs on 96-well plate</td>
<td>0.10</td>
</tr>
<tr>
<td>Cell on coverslips, Thermanox, TC plastic in 24-well plate</td>
<td>1.00</td>
</tr>
<tr>
<td>Cells on materials in 24-well plate</td>
<td>2.00</td>
</tr>
</tbody>
</table>

4. Incubate for 3 to 4 hours at 37°C, 5% CO₂

5. If using a 24-well plate, remove 2z 100μl aliquots from each well and transfer to a 96-well plate for reading. If the assay is being carried out in a 96-well plate, it can be read direct.

6. Read absorbance at 570nm with reference wavelength of 630nm on the Dynatech plate reader or measure fluorescence at an emission wavelength of 590nm (excitation wavelength 560nm).

7. If you wish to continue culturing the cells, remove the remaining Alamar Blue and wash the wells twice with PBS.

8. Add appropriate volume fresh culture medium to each well and return plates to incubator.
(6) MEASUREMENT OF CELL VIABILITY USING MTT

REFERENCES


PRINCIPLE

MTT((3-[4,5-dimethylthiazol-2-yl])-2,5,diilphenyl tetrazolium bromide) is converted to a blue formazan by mitochondrial dehydrogenases. The enzyme is present only in intact, living cells hence the blue colour produced should be proportional to the number of viable cells present.

REAGENTS

1. PHOSPHATE-BUFFERED SALINE(PBS)
2. MTT POWDER
   
   Sigma M2128 or M5665 (TC grade)
3. DIMETHYL SULPHOXIDE(DMSO)
4. Sigma d2650 ( TC grade)

METHOD (to be followed after the cells gave been treated)

1. Measure 10 ml PBS into a universal and warm at 37°C for 15 minutes.

2. Add 50mg MTT to the PBS, mix well and filter (remember to allow for some loss of volume in the filtration process when calculating the volume required). This solution should be prepared fresh ideally but is stable at 4°C for 1 week.

3. Add 10μl MTT solution to each well and incubate at 37°C for 4 hours. It is not necessary to remove the medium in which the cells have been incubating though you may prefer to do so.
APPENDIX I

4. Remove medium and excess MTT by inversion and blot plate carefully on paper.

5. Add 100μl DMSO to each well and shake plate for 5 minutes. Read absorbance on microplate reader (On Dynatech reader, ref wavelength is 630nm, test is 570nm).
(7) MEASUREMENT OF OSTEOCALCIN BY RADIOIMMUNOASSAY

REFERENCES


Stein GS and Lian JB (1993): Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocrine Reviews 14 424-442

PRINCIPLE

Osteocalcin (OC) molecules in standards or patient samples compete with $^{125}$I-labbelled OC for binding sites on and antibody to OC which is coated on the inside of the supplied tubes; this competition means that the higher the OC concentration in the standards or samples the lower the amount of radioactivity bound. Unbound OC is removes by decanting and washing and the residual radioactivity measured using a gamma counter.

HAZARD NOTES

You cannot carry out this assay until

- You have been on a radioactivity safety course.
- You have read the local safety rules on working with radioactivity and are familiar with disposal and monitoring procedures.

REAGENTS

1. "OSCATEST" OSTEOCALCIN KIT Brahms Diagnostica, Berlin

   The kit provides $^{125}$I-OC reagent (lyophilised) and buffer to reconstitute it, 100 antibody-coated tubes, zero serum (run as 0 standard and used to reconstitute standards and controls), six lyophilised standards and two lyophilised controls (a kit insert gives the expected levels). It also contains adhesive foil for sealing the tubes for incubation.

   It is important to order our kits so that they can be delivered in the week after radioactive labelling; $^{125}$I has a half-life of 60 days only so assays must be carried out as soon after receipt of kits as possible.
You can maximise the number of samples run with your kits by polling the reagents IF they have the same lot number and hence reducing the number of standard curves to be run.

METHOD

NB OC is a rather unstable protein and does not stand up to repeated freezing and thawing; you should try to ensure that assays for osteocalcin are carried out samples that have been frozen on collection and thawed for the OC assay only.

1. Remove samples and kit reagents from storage and allow to come to room temperature; mix thoroughly, avoiding foam formation.

2. Prepare worklist; you should allow 2 tubes for TOTAL COUNTS, labelled TC. Assign the remaining tube as follows: 1-12 standards in duplicate, 13-16 controls in duplicate. Samples should be run in duplicate starting from tube 17; run another set of controls in 4 tubes at the end of the assay and also about halfway through the samples if you are doing more than 100 tubes in total. Label the supplied coated tubes as appropriate.

3. Carefully remove the closures and stoppers from the lyophilised standards and controls and add 0.5ml zero serum to each one. Replace the rubber stoppers (make sure the right stopper goes in each vial!) and place vials on the Rolamixer for 10 minutes to allow reconstitution.

4. Pipette 50μl standards, controls and samples into their assigned tube.

5. Add 250μl reconstituted tracer to all tubes, including TC; stopper the TC tubers until reading the assay.

6. Mix all tubes briefly on a Whirlimix then seal with adhesive foil and incubate overnight at 4-8°C.

7. Allow the wash concentrate to come to room temperature then make it up to 500ml with distilled water.

8. Add 2ml wash solution to all tubers except TC then place in the decanting rack and carefully decant the solution down the sink in Room.14 Blot the tubers in a thick layer of paper briefly then repeat the washing procedure. Invert the tubes and drain them on absorbent paper for 5-10 minutes.

9. Count the tubes for 2 minutes using the gamma counter. Use the OSTC program
on the computer to plot the standard curve and calculate control and sample results.

10. Check the results for poor replicated and results that are out of range; samples should be rerun on the next assay. Check that the control values are within the quoted range and that they have not drifted within the assay.
Appendix II: Numerical data of the figures in the thesis:

Figure 2-1. ANOVA analysis in $^3$H-Thymidine incorporation assay of MSCs treated with different concentrations of FGF-2 at Day 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Results (CPM/μg DNA) (Mean ± S.D.)</th>
<th>Tukey's Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.49 ± 0.11</td>
<td>a</td>
</tr>
<tr>
<td>0.1 ng/ml</td>
<td>1.02 ± 0.24</td>
<td>b</td>
</tr>
<tr>
<td>1 ng/ml</td>
<td>3.05 ± 0.76</td>
<td>c</td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>5.27 ± 0.65</td>
<td>d</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>9.33 ± 0.96</td>
<td>e</td>
</tr>
</tbody>
</table>

Figure 2-2. ANOVA analysis in $^3$H-Thymidine incorporation assay of MSCs treated with different concentrations of FGF-2 at Day 7

<table>
<thead>
<tr>
<th>Groups</th>
<th>Results (CPM/μg DNA) (Mean ± S.D.)</th>
<th>Tukey's Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.76 ± 0.14</td>
<td>a</td>
</tr>
<tr>
<td>0.1 ng/ml</td>
<td>1.94 ± 0.39</td>
<td>b</td>
</tr>
<tr>
<td>1 ng/ml</td>
<td>4.88 ± 1.44</td>
<td>c</td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>6.73 ± 0.96</td>
<td>c</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>12.59 ± 0.78</td>
<td>d</td>
</tr>
</tbody>
</table>
### Figure 2-3. ANOVA analysis in $^3$H-Thymidine incorporation assay of MSCs treated with different concentrations of FGF-2 at Day 14

<table>
<thead>
<tr>
<th>Groups</th>
<th>Results (CPM/μg DNA) (Mean ± S.D.)</th>
<th>Tukey's Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.24 ± 0.24</td>
<td>a</td>
</tr>
<tr>
<td>0.1 ng/ml</td>
<td>3.69 ± 1.09</td>
<td>b</td>
</tr>
<tr>
<td>1 ng/ml</td>
<td>7.84 ± 0.96</td>
<td>c</td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>9.96 ± 0.73</td>
<td>c</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>15.83 ± 1.18</td>
<td>d</td>
</tr>
</tbody>
</table>

### Figure 2-4. ANOVA analysis in $^3$H-Thymidine incorporation assay of MSCs treated with different concentrations of FGF-2 at Day 19

<table>
<thead>
<tr>
<th>Groups</th>
<th>Results (CPM/μg DNA) (Mean ± S.D.)</th>
<th>Tukey's Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.97 ± 0.24</td>
<td>a</td>
</tr>
<tr>
<td>0.1 ng/ml</td>
<td>6.89 ± 0.79</td>
<td>b</td>
</tr>
<tr>
<td>1 ng/ml</td>
<td>11.34 ± 1.13</td>
<td>c</td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>20.87 ± 2.27</td>
<td>d</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>33.59 ± 1.89</td>
<td>e</td>
</tr>
</tbody>
</table>

### Figure 2-5. ANOVA analysis in alkaline phosphatase expression of MSCs treated with different concentrations of FGF-2 at Day 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Results (nmol p-nitrophenol/hour/μg DNA) (Mean ± S.D.)</th>
<th>Tukey's Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.78 ± 0.06</td>
<td>a</td>
</tr>
<tr>
<td>0.1 ng/ml</td>
<td>0.84 ± 0.11</td>
<td>a</td>
</tr>
<tr>
<td>1 ng/ml</td>
<td>0.83 ± 0.09</td>
<td>a</td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>0.82 ± 0.04</td>
<td>a</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>0.81 ± 0.07</td>
<td>a</td>
</tr>
</tbody>
</table>
### APPENDIX II

Figure 2-6. ANOVA analysis in alkaline phosphatase expression of MSCs treated with different concentrations of FGF-2 at Day 7

<table>
<thead>
<tr>
<th>Groups</th>
<th>Results (nmol p-nitrophenol/hour/μg DNA) (Mean ± S.D.)</th>
<th>Tukey’s Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.77 ± 0.17</td>
<td>a</td>
</tr>
<tr>
<td>0.1 ng/ml</td>
<td>0.79 ± 0.09</td>
<td>a</td>
</tr>
<tr>
<td>1 ng/ml</td>
<td>0.82 ± 0.06</td>
<td>a</td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>0.85 ± 0.06</td>
<td>a</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>0.83 ± 0.14</td>
<td>a</td>
</tr>
</tbody>
</table>

Figure 2-7. ANOVA analysis in alkaline phosphatase expression of MSCs treated with different concentrations of FGF-2 at Day 14

<table>
<thead>
<tr>
<th>Groups</th>
<th>Results (nmol p-nitrophenol/hour/μg DNA) (Mean ± S.D.)</th>
<th>Tukey’s Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.72 ± 0.09</td>
<td>a</td>
</tr>
<tr>
<td>0.1 ng/ml</td>
<td>0.77 ± 0.13</td>
<td>a</td>
</tr>
<tr>
<td>1 ng/ml</td>
<td>0.75 ± 0.04</td>
<td>a</td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>0.81 ± 0.06</td>
<td>a</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>0.80 ± 0.11</td>
<td>a</td>
</tr>
</tbody>
</table>

Figure 2-8. ANOVA analysis in alkaline phosphatase expression of MSCs treated with different concentrations of FGF-2 at Day 19

<table>
<thead>
<tr>
<th>Groups</th>
<th>Results (nmol p-nitrophenol/hour/μg DNA) (Mean ± S.D.)</th>
<th>Tukey’s Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.74 ± 0.19</td>
<td>a</td>
</tr>
<tr>
<td>0.1 ng/ml</td>
<td>0.81 ± 0.06</td>
<td>a</td>
</tr>
<tr>
<td>1 ng/ml</td>
<td>0.76 ± 0.14</td>
<td>a</td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>0.79 ± 0.09</td>
<td>a</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>0.80 ± 0.110</td>
<td>a</td>
</tr>
</tbody>
</table>
Figure 2-14. ANOVA analysis in $^3$H-Thymidine incorporation of MSCs treated with different exposure time of FGF-2 at Day 7

<table>
<thead>
<tr>
<th>Groups</th>
<th>Results (CPM/μg DNA) (Mean ± S.D.)</th>
<th>Tukey's Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.687 ± 0.443</td>
<td>a</td>
</tr>
<tr>
<td>4 hours</td>
<td>5.962 ± 0.756</td>
<td>b</td>
</tr>
<tr>
<td>6 hours</td>
<td>8.814 ± 1.152</td>
<td>c</td>
</tr>
<tr>
<td>16 hours</td>
<td>12.380 ± 0.844</td>
<td>d</td>
</tr>
<tr>
<td>24 hours</td>
<td>15.688 ± 0.567</td>
<td>e</td>
</tr>
</tbody>
</table>

Figure 5-10. Bone mineral density in the osteotomy gaps of the rats treated and not treated with chemotherapy

<table>
<thead>
<tr>
<th></th>
<th>Control Group (g/cm$^3$) (Mean ± S.D.)</th>
<th>Chemo Group(g/cm$^3$) (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>0.108 ± 0.018</td>
<td>0.073 ± 0.008*</td>
</tr>
<tr>
<td>Week 2</td>
<td>0.163 ± 0.017</td>
<td>0.097 ± 0.007</td>
</tr>
<tr>
<td>Week 3</td>
<td>0.184 ± 0.017</td>
<td>0.113 ± 0.012</td>
</tr>
<tr>
<td>Week 4</td>
<td>0.194 ± 0.015</td>
<td>0.120 ± 0.009</td>
</tr>
<tr>
<td>Week 5</td>
<td>0.202 ± 0.019</td>
<td>0.127 ± 0.009</td>
</tr>
</tbody>
</table>

* p < 0.01
Figure 6-8. Alamar blue assay of MSCs in fibrin glue

<table>
<thead>
<tr>
<th>Groups</th>
<th>Results (percentage of control) (Mean ± S.D.)</th>
<th>Tukey's Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hours</td>
<td>87.01 ± 2.86</td>
<td>a</td>
</tr>
<tr>
<td>24 hours</td>
<td>76.25 ± 3.76</td>
<td>a</td>
</tr>
<tr>
<td>48 hours</td>
<td>80.16 ± 4.17</td>
<td>a</td>
</tr>
<tr>
<td>72 hours</td>
<td>80.30 ± 5.24</td>
<td>a</td>
</tr>
<tr>
<td>96 hours</td>
<td>84.09 ± 5.56</td>
<td>a</td>
</tr>
</tbody>
</table>

Figure 6-9. ^H-Thymidine incorporation assay of MSCs in fibrin glue

<table>
<thead>
<tr>
<th>Groups</th>
<th>Results (CPM/μg DNA) (Mean ± S.D.)</th>
<th>Tukey's Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>22.19 ± 3.44</td>
<td>a</td>
</tr>
<tr>
<td>48 hours</td>
<td>19.80 ± 4.17</td>
<td>a</td>
</tr>
<tr>
<td>72 hours</td>
<td>21.04 ± 3.69</td>
<td>a</td>
</tr>
<tr>
<td>96 hours</td>
<td>21.60 ± 3.46</td>
<td>a</td>
</tr>
</tbody>
</table>
**APPENDIX II**

Fig 6-23. ANOVA analysis of DEXA scan results (g/cm\(^3\)) at the osteotomy gap three weeks after the operation

<table>
<thead>
<tr>
<th>Groups</th>
<th>DEXA scan results (g/cm(^3)) (mean ± S.D.)</th>
<th>Tukey’s analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.132 ± 0.009</td>
<td>b</td>
</tr>
<tr>
<td>2</td>
<td>0.143 ± 0.010</td>
<td>b</td>
</tr>
<tr>
<td>3</td>
<td>0.169 ± 0.012</td>
<td>c</td>
</tr>
<tr>
<td>4</td>
<td>0.099 ± 0.007</td>
<td>a</td>
</tr>
<tr>
<td>5</td>
<td>0.114 ± 0.009</td>
<td>a</td>
</tr>
<tr>
<td>6</td>
<td>0.161 ± 0.008</td>
<td>c</td>
</tr>
</tbody>
</table>

Fig 6-24. ANOVA analysis of DEXA scan results (g/cm\(^3\)) at the osteotomy gap five weeks after the operation

<table>
<thead>
<tr>
<th>Groups</th>
<th>DEXA scan results (g/cm(^3)) (mean ± S.D.)</th>
<th>Tukey’s analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.186 ± 0.011</td>
<td>b</td>
</tr>
<tr>
<td>2</td>
<td>0.193 ± 0.007</td>
<td>b</td>
</tr>
<tr>
<td>3</td>
<td>0.221 ± 0.011</td>
<td>c</td>
</tr>
<tr>
<td>4</td>
<td>0.137 ± 0.012</td>
<td>a</td>
</tr>
<tr>
<td>5</td>
<td>0.148 ± 0.015</td>
<td>a</td>
</tr>
<tr>
<td>6</td>
<td>0.214 ± 0.013</td>
<td>c</td>
</tr>
</tbody>
</table>
Figure 6-31. Quantitative histological analysis of percentage bone formation at the osteotomy gap

<table>
<thead>
<tr>
<th>Groups</th>
<th>Percentage bone formation (mean ± S.D.)</th>
<th>Tukey’s analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38.51 ± 2.57</td>
<td>b</td>
</tr>
<tr>
<td>2</td>
<td>36.42 ± 3.13</td>
<td>b</td>
</tr>
<tr>
<td>3</td>
<td>47.70 ± 2.89</td>
<td>c</td>
</tr>
<tr>
<td>4</td>
<td>21.51 ± 4.03</td>
<td>a</td>
</tr>
<tr>
<td>5</td>
<td>23.62 ± 3.76</td>
<td>a</td>
</tr>
<tr>
<td>6</td>
<td>46.77 ± 3.49</td>
<td>c</td>
</tr>
</tbody>
</table>