ROYAL FREE THESES 1995

Iodised Oil in the Management of Hepatocellular Carcinoma

An experimental and clinical investigation

Satyajit Bhattacharya   MS (Bombay), FRCS (Edinburgh)
Royal Free Hospital and School of Medicine, London

ProQuest Number: 10046203

ProQuest 10046203

07751

This thesis is dedicated to
my wife Shanti

Abstract

Lipiodol (iodised poppyseed oil) administered into the hepatic artery is selectively retained by primary hepatocellular carcinomas (HCCs) for prolonged periods, and targeted therapy with Lipiodol conjugated to a cytotoxic drug or radioisotope has been used to treat these tumours. The mechanism of Lipiodol retention has remained unclear so far. A clear understanding of why Lipiodol is retained by tumours is essential to improving the efficacy of Lipiodol-targeted therapies.

To investigate any cellular role in this process, the interaction of Lipiodol with tumour and endothelial cells was studied *in vitro* in cell cultures and *in vivo* in human HCCs. Cultures of HepG2 (a human liver tumour cell line) and HUVECs (human umbilical vein endothelial cells, which share phenotypic markers with the endothelium in HCCs) were exposed to 1%, 2% and 4% Lipiodol in culture medium for 4, 8, 24 and 32 hours. Cell monolayers were stained for Lipiodol by a selective silver nitrate impregnation technique. All tumour and endothelial cultures demonstrated incorporation of Lipiodol into the cells. Intracellular Lipiodol uptake was quantitated by computer-assisted image analysis of the optical density of silver-stained monolayers. A significant increase in optical density was noted with every concentration of Lipiodol (n=10; p<0.05, Wilcoxon Signed Rank test). HepG2 cells demonstrated a slow rate of uptake initially, followed by progressive intracellular accumulation. On the other hand, HUVECs demonstrated rapid initial uptake, but subsequently the optical densities diminished, indicating that the Lipiodol had been excreted or metabolised by the cells. Electron microscopy of HepG2 cells demonstrated membrane-bound lipid vesicles in the cytoplasm, suggestive of uptake by pinocytosis. The effect of Lipiodol on tumour cells was assessed with respect to cell viability (Trypan Blue exclusion and media Lactate Dehydrogenase levels), cell numbers and protein synthesis (^{3}H-labelled Leucine incorporation). Lipiodol had no significant effect on any of these parameters.

Histologic assessment of Lipiodol retention in HCCs was performed on surgically resected tumours (n = 8) administered pre-operative arterial Lipiodol-Epirubicin and incidental HCCs (n = 15) discovered in cirrhotic

3

livers removed at orthotopic liver transplantation, wherein Lipiodol had been arterially administered at prior angiography. All tumour sections were stained by silver nitrate impregnation. Light and electron microscopy confirmed Lipiodol incorporation by tumour cells and by endothelial cells lining tumour vessels. Intracellular Lipiodol was also demonstrated in one incidental HCC perfused with Lipiodol *ex vivo* immediately after removal from the body, which would suggest that cellular incorporation of Lipiodol occurs rapidly after administration.

In the clinical context, it is as yet unclear which is the ideal therapeutic agent for conjugation to Lipiodol. There have been few comparative studies of the available cytotoxic drug regimens, and no reported comparisons of Lipiodol-targeted chemotherapy with Lipiodol-targeted radiotherapy. This study assessed 95 patients with unresectable HCC confined to the liver. Sixty-nine were treated with Lipiodol-Epirubicin emulsion [median age 61 yrs; Cirrhotics - Child's grade A27, B27, C7; Tumour stage (Okuda) I 14, II 37, III 18; Epirubicin dose 75 mg/m^2] and 26 received Lipiodol-^{131}I [median age 64 yrs; Cirrhotics - Child's grade A13, B5, C0; Tumour stage I 6, II 19, III 1; Dose 750-1050 MBq]. The last 28 patients (17 Epirubicin, 11 ^{131}I) were treated within a prospective randomised trial. Bolus drug or isotope was injected into the hepatic artery by transfemoral cannulation. Lipiodol and ^{131}I uptake were gauged by 10th day CT and 48-hr scintiscan. Tumour size at 2 months remained static or diminished partially in 21/38 Epirubicin recipients (55%) and 15/22 ^{131}I recipients (68%). Treatments were repeated 2 monthly when clinically indicated. Cumulative survival at 6, 12 and 24 mths was 40%, 25% and 6% with Epirubicin, and 58%, 25% and 0% with ^{131}I; 30-days mortality was 11% and 15% respectively. Comparison with historical controls indicated a survival benefit in Stage I/II disease. Similar findings were recorded in the subset of 28 patients in the randomised controlled trial.

In conclusion, liver tumour cells and endothelial cells exposed to Lipiodol *in vitro* incorporate it, possibly by pinocytosis, without suffering obvious adverse effects. Similar mechanisms may operate *in vivo*. Clinically, patients with Stage I/II HCC receiving Lipiodol-Epirubicin or Lipiodol-^{131}I show good localisation, acceptable toxicity, and comparable survival benefit at 6 & 12 months with either modality.

Acknowledgements

5

Statement of originality

This thesis is based on original work carried out by the author from 1992 to 1994 in the University Department of Surgery at the Royal Free Hospital School of Medicine.

The experimental studies described in this thesis (Chapters 2, 3 and 4) were designed by the author. The author was solely responsible for collection of material, conduct of the experiments, and analysis of the data.

The clinical study (Chapter 5) was designed in liaison with the hospital departments concerned. All patients included in the prospective randomised trial were initially assessed by the author, who was responsible for organisation of admission, angiography, CT scans and other scintigraphy. Retrieval from case notes of data pertaining to follow-up and investigations was carried out by the author. Prior to commencement of the prospective study, a proportion of the patients were followed up and separately studied by a research colleague. Where appropriate, this has been acknowledged in the text and resulting publications have been listed. The author has performed the statistical analyses pertaining to the clinical study, and prepared the manuscript and accompanying figures.

CONTENTS

10

11

Appendices

List of figures

List of tables

List of abbreviations

HCC	Hepatocellular carcinoma
AFP	Alpha foetoprotein
CT	Computed tomography
TAE	Trans-arterial embolisation
TOCE	Trans-arterial oily chemo-embolisation
SMANCS	Styrene - maleic acid - neocarzinostatin
HUVECs	Human umbilical vein endothelial cells
DMEM	Dulbecco's modified Eagle's medium
PBS	Phophate buffered saline
FCS	Foetal calf serum
NBCS	New-born calf serum
IOD	Integrated optical density
LDH	Lactate dehydrogenase
DPM	Disintegrations per minute
OLT	Orthotopic liver transplantation

Chapter 1

USE OF IODISED OIL IN THE DIAGNOSIS AND TREATMENT OF HEPATOCELLULAR CARCINOMA: A REVIEW

1.1   HEPATOCELLULAR CARCINOMA: AN OVERVIEW

Epidemiology

Incidence: Geographic variations

Age, Sex and Race

Aetiologic Factors

Cirrhosis

Hepatitis B and C viruses

Other factors

Pathology

Presentation and diagnosis

Clinical features

Diagnostic tests

Natural history of hepatocellular carcinoma: Staging and prognosis

Staging classifications

Prognosis in relation to tumour stage

Surgical treatment of hepatocellular carcinoma

Resection

Transplantation

1.2   TREATMENT OF ADVANCED HEPATOCELLULAR CARCINOMA:
A BACKGROUND TO THE USE OF IODISED OIL

Therapeutic options in unresectable hepatocellular carcinoma

Systemic chemotherapy and external beam irradiation

"Local" treatments: Alcohol injection, Laser ablation and Cryotherapy

Immunotherapy

Intra-arterial chemotherapy with cytotoxic agents

Devascularisation

A brief history of the use of iodised oil

1.3   PHYSICAL AND CHEMICAL PROPERTIES OF LIPIODOL

Physical properties and chemical composition

Drug-Lipiodol emulsions

Use of diatrizoates

1.1   HEPATOCELLULAR CARCINOMA: AN OVERVIEW

Epidemiology

Incidence: Geographic variations

The first known reference to cancer of the liver as a pathological entity is to be found in the writings of Galen and Aretaeus, from the 2nd century AD (1). Virchow (1821-1902) was one of the first to draw a clear distinction between primary and metastatic cancers of the liver. Even three to four decades ago, primary hepatocellular carcinoma (HCC) was regarded as a fortunately rare tumour, though uniformly fatal. Today it

is well recognised as one of the commonest malignancies worldwide. Large geographic variations have been reported in the incidence of HCC. Annual incidence of primary liver cancer per 100,000 males ranges from 2.7 in the US and 3.0 in the UK, to 4.6 in Japan, 5.5 in Singapore, 14.2 in South Africa, 17.0 in China and 98.2 in Mozambique (2). Incidence of HCC at autopsy varies from <1% in Europe and North America to >5% in Hong Kong, Singapore and Taiwan. While HCC comprises less than 2.5% of all cancers in Europe and North America, the incidence rises to between 16 and 19% in Singapore and Taiwan, and in parts of Southern Africa it may comprise close to 50% of all malignancies (3, 4).

Age, Sex and Race

In Southern Africa, the peak incidence is around the age of 40 years and in Asia, between 40 and 60 years. In areas of low incidence (such as the UK) the highest incidence is in the 80's (5, 6). Men are affected 2 to 8 times more commonly than women. In mixed populations certain races are found to have a higher incidence of HCC, in particular, the black population in South Africa and the ethnic Chinese in South-east Asia. A study of autopsies in the South African Bantu revealed HCC in 4 out of 5 cases (7).

Aetiologic Factors

Cirrhosis

The frequency of HCC in cirrhotic livers at autopsy ranges from over 30% in South Africa and Indonesia, to 10-20% in India, UK and North America. Conversely, figures from the UK indicate that 68-74% of patients with HCC have cirrhosis (8, 9). While cirrhosis may be pre-malignant irrespective of its etiology, the incidence of HCC is higher in certain types of cirrhosis, ranging from 42% in HBV-related chronic active hepatitis and 36% in haemochromatosis, to 3% in primary biliary cirrhosis (9). Liver cell dysplasia may be an intermediate step (10) and is present in 60% of cirrhotic livers harbouring HCC as compared to 10% of non-cirrhotic livers.

Hepatitis B and C viruses (HBV and HCV)

Worldwide, incidence of HBV carriage corresponds to the frequency of primary liver cancer. Individuals who harbour the Hepatitis B surface antigen (HBsAg positive) are 390 times more likely to get HCC than those who are HBsAg negative (11). In patients with HCC, even if HBsAg is absent in the blood, anti-HBc (i.e. antibody to the HBV core antigen) is often present (12) and HBV DNA sequences can be detected in tumour biopsies by the polymerase chain reaction (PCR). It has been proposed that the viral DNA integrates with the host DNA, followed by changes in gene expression (13). This, possibly coupled with interaction with co-carcinogens, leads to clonal proliferation of these hepatocytes. Animal parallels exist in Woodchucks and Peking Ducks, which commonly develop HCC when infected with HBV-like (HEPADNA) viruses (14).

High associations between chronic HCV infection and HCC have been reported from Japan (15), Italy (16), Spain (17), South Africa (18) and the US. The incidence of HCC in HCV carriers has been reported as four times higher than in HBV carriers. The mechanism of carcinogenesis remains unclear as HCV is an RNAvirus without reverse transcriptase, and does not merge with the host genome. It is possible that HCC follows nodular regeneration in these livers, or as a result of an interaction between HBV and HCV.

Other factors

There is a fourfold increased risk of HCC amongst alcoholics in Europe and North America (19). Cirrhosis is always present. Alcohol may be a cocarcinogen with HBV, though HCC can develop in alcoholics without any evidence of HBV infection. In animals, chemicals such as P-dimethyl-amino-azobenzene, nitrosamines, aflatoxin and senecio alkaloids have been reported as carcinogenic. In man, the mycotoxin Aflatoxin, produced by Aspergillus flavus, a mould contaminating groundnuts and grains stored has been linked to HCC (2, 20). Mutations of p53 (a tumour suppressor gene on the short arm of chromosome 17) have been found in several human cancers. In HCCs from Africa and South Asia, a "hot spot" has been noted in codon 49 which shows geographic variations and has been linked to aflatoxin intake (21).

25
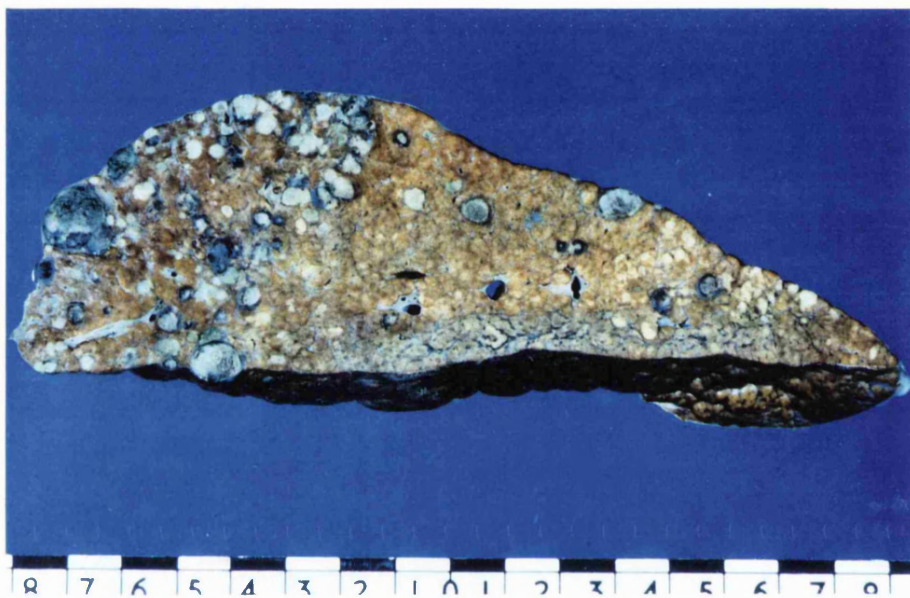
Figure 1.1: Multifocal nodular HCC in a cirrhotic liver
Macroscopic appearance

Pathology

HCCs may be simply classified as massive, nodular or diffuse, *or* as encapsulated or infiltrative. The lesions are frequently multifocal (Figure 1.1). Kojiro and Nakashima (1) have proposed a classification into the following categories:

1. Infiltrative (33%) - with a tendency to intrahepatic spread
2. Expansive or nodular (18%) which may be a solitary nodule or multi-nodular
3. Mixed infiltrative and expansive (33%) which again may be uni- or multi-nodular
4. Diffuse (5%) - only seen in cirrhotic livers

Geographic variations have been noted in these anatomic features, and a high incidence of encapsulated HCCs has reported in Japan (22).

The histological picture ranges from well-diferentiated lesions with cells resembling hepatocytes, arranged in cords or nests, to poorly differentiated lesions with anaplastic tumour cells. There may be evidence of bile production, but biliary canaliculi are typically absent (23).

Presentation and diagnosis

Clinical features

HCC often presents in an insidious manner. Berman aptly described the possible presentations of HCC as "Frank" (hepatomegaly or a palpable discrete mass; right hypochondrial discomfort), "Febrile" (pyrexia, weight loss, anorexia), "Icteric", "Acute" (presenting with acute abdominal pain), "Metastatic" and "Occult" (7). Clinical examination may reveal ascites and a friction rub or bruit over the tumour. In cirrhotics, the initial symptoms of HCC may be misinterpreted as indicative of progression of the underlying pathology, leading to delay in tumour diagnosis. It has therefore been suggested that cirrhotics should be offered regular screening with ultrasonography and tumour marker (alpha foeto protein) estimation, to allow early diagnosis of HCC (24)

The imaging modalities used in the diagnosis of HCC have been discussed subsequently in this chapter, in the context of the diagnostic uses of iodised oil.

Alpha foeto protein (AFP) is a α_1 globulin normally present in the foetus but not in adults. It is likely that in the course of neoplastic transformation into HCC, the coding for synthesis of this protein gets de-repressed, as high levels of AFP are present in 70-90% of patients with HCC, and AFP estimation has now found acceptance as a screening procedure for HCC (25). Titres may also be raised in patients with embryonal yolk sac tumours, and to a lesser extent in cases of acute or chronic viral hepatitis and secondary tumours of the liver. It has been suggested that a high initial titre of AFP is a poor prognostic indicator (26). The AFP level generally falls after surgical resection, and a subsequent rise is an indicator of tumour recurrence. Other tumour markers have been investigated - such as des gamma carboxy prothrombin (DCP), an abnormal prothrombin induced by Vitamin K absence or antagonism in tumour cells, and α-L-fucosidase - but not found widespread acceptance as yet (27, 28).

Histologic confirmation of the diagnosis necessitates a needle liver biopsy. This may be done by the conventional percutaneous approach, with a Menghini 'Trucut' needle. The use of ultrasound or CT imaging at the time of biopsy adds to the diagnostic accuracy. In selected cases, a transjugular biopsy may be attempted. Liver biopsy carries an overall mortality of 0.01% (2), and potential complications include haemorrhage (intra-peritoneal, intra-thoracic or intra-hepatic), haemobilia, biliary peritonitis, and inadvertent dissemination of tumour along the biopsy track. Fine needle aspiration cytology may be performed instead of a Trucut biopsy, to add to the safety.

Natural history of hepatocellular carcinoma: Staging and prognosis

Staging classifications

In 1985 Okuda and colleagues devised a staging scheme for HCC (Table 1.1) and analysed the natural history of the tumour in 850 patients in relation to the stage of their disease (29). The staging system (which is now widely accepted and has the advantage of simplicity) is summarised below, and the TNM staging classification (30) is provided for comparison (Table 1.2).

Table 1.1

Okuda Staging System for HCC

Parameters	Stages
Tumour size > 50% of liver: 1 point Ascites: 1 point Albumin < 30 g/l: 1 point Bilirubin > 30 μmol/l: 1 point	Stage I: 0 points Stage II: 1-2 points Stage III: 3-4 points

Table 1.2

pTNM Pathologic Staging Classification for HCC

Stage	T	N	M
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1	N1	M0
	T2	N1	M0
	T3	N0/N1	M0
Stage IV A	T4	Any N	M0
Stage IV B	Any T	Any N	M1

T1:   Solitary, ≤ 2cm, without vascular invasion
T2:   Solitary, ≤ 2cm, with vascular invasion *or* Multiple, one lobe, ≤ 2cm, without vascular invasion *or* Solitary, > 2cm, without vascular invasion
T3:   Solitary, > 2cm, with vascular invasion *or* Multiple, one lobe, > 2cm, with or without vascular invasion
T4:   Multiple, > one lobe *or* Invasion of major branch of portal or hepatic veins
N1:   Regional nodes
M1:   Distant metastasis

Prognosis

The overall prognosis in HCC is dismal. Without therapy, the median survival reported by Okuda (29) is 1.6 months (Table 1.3). Similar figures have been reported by Lee et al (31). A French study that followed 30 untreated patients with HCC reported no survivors at one year (32).

Table 1.3

Natural history of HCC

Median survival in 850 patients,
stratified according to tumour stage (29)

Okuda Stage	No treatment (229 patients)	Surgery (157 patients)	Medical therapy (464 patients)
Stage I	8.3 months	25.6 months	9.4 months
Stage II	2.0 months	12.2 months	3.5 months
Stage III	0.7 months	-	1.6 months
Overall	1.6 months	21.6 months	-

Surgical treatment of hepatocellular carcinoma

Resection

Surgical resection of the tumour is currently considered the treatment of choice for HCC at many centres. However, the majority of patients have unresectable tumour at the time of presentation. The Liver Cancer Study Group of Japan, in two large surveys of primary liver cancer (2411 cases from 1968-77 and 12887 cases from 1982-85) reported resectability rates of 11.9% and 18.1% (33, 34). Okuda, in his series of 850 patients had 157 (18.5%) with resectable lesions (29). At the Mayo Clinic, of 123 patients with HCC seen between 1972 and 1982, 34 (28%) were offered surgical resection (35). In South Africa, less than 2% may be resectable (36). Age, infirmity, large tumour size, multifocal lesions involving both lobes, involvement of major vascular structures in or around the liver, extrahepatic tumour spread, and the presence of concomitant

cirrhosis leading to poor hepatic reserve are the common reasons why a tumour may be unresectable. Hepatic resection for HCC carries a peri-operative mortality rate of around 5% (37), though Adson, in a review of 13 Western series, reports a range from 5 to 42% (38). In cirrhotics, the mortality rate rises significantly, to between 10 and 50% (38, 39). Five-year survival following "curative" resection has been reported at between 12 and 20% in Asian series (29, 31, 40), and between 16 and 46% in Western series (35, 38).

Transplantation

Orthotopic liver transplantation for unresectable HCC confined to the liver has yielded poor long-term survival compared to transplantation for benign conditions, with 39% recurrence and 2-year and 5-year survivals of 30% and 18% (41). But when compared to hepatic resection for HCC, the survival figures are comparable for each tumour stage (42, 43). When HCC was associated with cirrhosis, the survival rates after transplantation were significantly better than after resection (42). It has been suggested that the indication for transplantation should be extended.beyond unresectable tumours to include resectable tumours arising in cirrhotic livers. In patients with small uninodular or binodular tumours (<3 cm) transplantation has been reported to yield significantly better results than resection (43).

1.2   TREATMENT OF ADVANCED HEPATOCELLULAR CARCINOMA: A BACKGROUND TO THE USE OF IODISED OIL

Therapeutic options in unresectable hepatocellular carcinoma

Systemic chemotherapy and external beam irradiation

The therapeutic modalities available to treat patients with advanced HCC, unsuitable for resection or transplantation, are listed in Table 1.4. Conventional systemic chemotherapy with cytotoxic agents, singly or in combination, has been associated with unimpressive degrees of tumour

Table 1.4

Therapeutic modalities that may be used in advanced HCC

Systemic chemotherapy

External beam irradiation

Local treatments
  Percutaneous alcohol injection into tumour
  Laser hyperthermia
  Cryotherapy

Immunotherapy
  Anti-ferritin antibodies

Devascularisation
  Trans-arterial embolisation (TAE)

Intra-arterial chemotherapy
  Cytotoxic agent(s)

Intra-arterial Lipiodol-targeted chemotherapy
  Cytotoxic agent(s) with Lipiodol
  Cytotoxic agent(s) with Lipiodol, plus embolisation

Intra-arterial radiotherapy
  Iodine-131 Lipiodol
  Yttrium-90 microspheres

response, and long term survival has been dismal (44-47). Its role as an additional therapy administered in conjunction with loco-regional treatments remains to be clarified. External beam irradiation to hepatic lesions is limited by the radiosensitivity of the liver parenchyma. Whole liver irradiation at 35 Gy is associated with a high incidence of radiation hepatitis. An acceptable maximum dose for a healthy liver would range between 20 and 35 Gy, delivered in daily fractions of 200 cGy (48), but in a cirrhotic liver even such doses are associated with a high incidence of hepatic failure (49).

For small tumours less than 3 cm in size, percutaneous injection of alcohol directly into the lesion under radiologic guidance has been attempted with encouraging short-term results (50). This leads to coagulative necrosis of the tumour, and the technique has been advocated particularly for patients with "resectable" lesions but who are unfit for surgery. It has been suggested that survival in patients with small HCCs treated by this modality equals that achieved by surgical resection (49). However, tumour recurrence from persisting malignant cells remains a potential problem, and it remains to be clarified how many injections should be administered to a given lesion, and what volumes of alcohol should be used. Complications of this procedure include pain, fever and haemorrhage. Long term data on this treatment is still emerging (51, 52). Other "local" treatments that have been reported but are yet to find widespread acceptance include cryotherapy, which has been used in treating superficial tumour deposits at laparotomy (53, 54) and laser hyperthermia (55). Direct intratumoral injection of radioactive Yttrium (^{90}Y) in animals has been associated with a reduction in tumour size and prolonged survival (56). Iridium (^{192}Ir) impantation is used for palliation of malignant biliary obstruction (57), but its low beta energy and range makes it a poor choice for intratumoral injection in the liver.

Immunotherapy

Monoclonal antibodies specific for HCC have been raised with a degree of success. Intravenous administration of radiolabelled antibody has shown good tumour localisation (58), but autoradiography of the treated tissues revealed patchy localisation of the isotope. HCC cells have a high level of ferritin, and trials of polyclonal anti-ferritin antibodies labelled with ^{131}I or ^{90}Y have yielded impressive remission rates (59, 60). However, some of the protocols used other treatment modalities at the same time, and it is difficult to determine the relative contribution of each component. Cross-reactivity with ferritin in other organs and marrow toxicity caused by ^{90}Y are among the problems associated with this technique (61). Monoclonal anti-ferritin has been shown to be superior to the polyclonal antibody in an animal model (62).

Intra-arterial chemotherapy with cytotoxic agents

The failure to achieve impressive results with systemic and local treatments has led to an increasing interest in targeted therapies delivered via the hepatic artery. Selective hepatic arterial infusion of cytotoxic agents alone has been associated with a higher response rate and better survival figures than systemic therapy (44). However, while this has the potential advantage of achieving a better response with a lower dose (and consequently, less dose-related morbidity), it does not ensure prolonged retention and gradual release of the drug within the tumour.

Devascularisation

Transcatheter arterial embolisation (TAE) of the hepatic arterial bed is an approach that has been tried with some success (63). The rationale for this is based on the observation that HCCs receive their blood supply predominantly from the hepatic artery (64), and devascularisation of the tumour may be achieved by hepatic arterial ligation. Substances used for TAE include gelatin sponge (gelfoam), polyvinyl alcohol (Ivalon), steel coils, degradable starch microspheres, and plastic or metal microspheres (65). TAE has been used effectively for palliation of unresectable or recurrent tumours (65, 66). However, malignant cells in the tumour are not fully eradicated, leading to eventual recurrence. The procedure is generally accepted to be contraindicated in patients with occlusion of the main trunk of the portal vein, in view of the risk of massive hepatic necrosis following the loss of arterial and portal inflow. TAE is frequently associated with a "post-embolisation syndrome" involving pain, fever and malaise, which is usually self-limiting (67). More serious complications include infection compounded by septicaemia, local complications secondary to misplaced embolic material, such as cholecystitis, pancreatitis, gastroduodenal haemorrhage and, in 1% of patients, rupture of the tumour (68). The use of TAE for debulking prior to surgical resection is controversial (69-71). In several of the studies reporting on the use of TAE, the embolic material was additionally impregnated with an anticancer agent, such as doxorubicin or mitomycin, making it difficult to evaluate the relative contributions of the embolic and cytotoxic effects towards the improvement in survival.

A brief history of the use of iodised oil

Sicard and Forestier, in 1923, were the first to report the use of iodised oil as a contrast medium (72). They administered iodised poppyseed oil into the antecubital vein to observe flow through the heart and pulmonary arteries. Since then, iodised oil has been used as a contrast medium in pedal lymphangiography, bronchography, hystero-salpingography, sinograms and fistulograms (73). In 1966, Idezuki et al injected iodised oil into the portal vein of patients with liver cancer, and visualised the tumours radiolucent defects in an opacified liver (74). In 1979, Nakakuma and colleagues first reported the selective retention of iodised oil in foci of HCC, following its injection into the hepatic artery (75). It is now well established that iodised oil is retained by hepatocellular carcinomas for periods ranging from several weeks to over a year, while it is cleared from normal liver parenchyma within 7 days (76-78). This property of Lipiodol has been utilised extensively in diagnostic imaging and in selectively delivering therapeutic agents to these tumours.

1.3   PHYSICAL AND CHEMICAL PROPERTIES OF LIPIODOL

Physical properties and chemical composition

The commonly available preparation of iodised oil is Lipiodol™. Lipiodol Ultra Fluid (Laboratoire Guerbet, Roissy Charles de Gaulle, France) is a straw-coloured oily fluid, with a specific gravity between 1.28 and 1.30, and an iodine content of 37 to 39% w/w (B.P.) (Figure 1.2). It is insoluble in water and dissolves in organic solvents such as acetone, chloroform and ether. It decomposes on exposure to sunlight and should be stored at temperatures below 25°C.
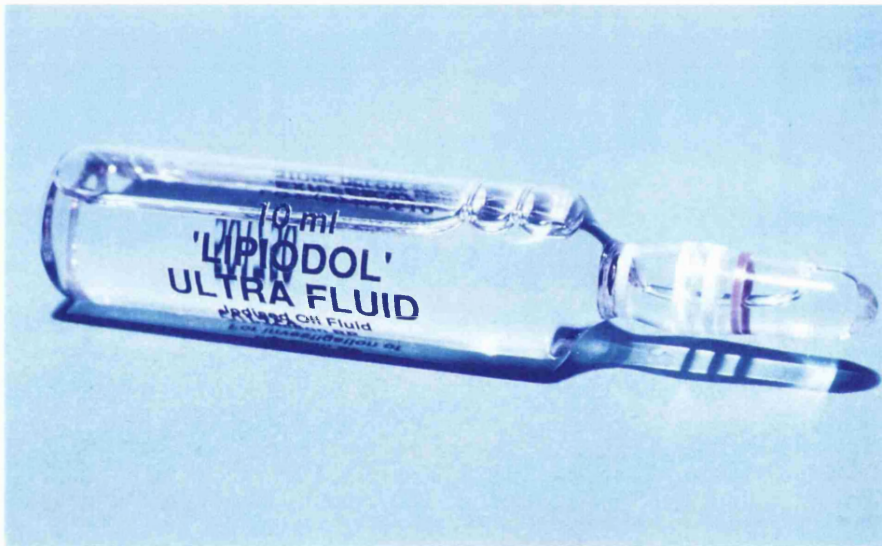
Figure 1.2: Lipiodol Ultra-Fluid

Lipiodol is manufactured by ethyl trans-esterification of poppyseed oil, and largely consists of mono-, di- and tri-iodinated ethyl esters of four fatty acids, namely Linoleic (73%), Oleic (14%), Palmitic (9%) and Stearic (3%) acid (79).

Lipiodol is probably not unique in its property of being retained by liver tumours. Iwai et al (80) have demonstrated selective uptake of other lipids such as linoleic acid, olive oil, tea seed oil and medium-chain triglyceride by liver tumours in an animal model. However, Lipiodol has the advantage of being radio-opaque and therefore useful as a contrast medium. Also, as it has been in use for several decades, its adverse effects (or absence of adverse effects) are well documented.

Drug-Lipiodol emulsions

Lipiodol is immiscible with water. Mechanical agitation of a water-Lipiodol mixture (by ultrasonication or syringe-to-syringe transfer) creates an oil-in-water emulsion, but the two phases tend to separate after a time. This led to initial concern about the efficacy of Lipiodol as a vehicle for cytotoxic chemotherapeutic agents. Most of the drug formulations available for parenteral use are water-soluble preparations that do not mix well with Lipiodol, and it was thought possible that though Lipiodol localises selectively in tumours, the cytotoxic agent may separate from it in vivo.

Use of diatrizoates

Many centres therefore now use the technique of 'Lipiodolisation', proposed by Kanematsu et al (81), whereby the anti-cancer drug is first dissolved in Urografin (73), an aqueous contrast medium containing meglumine diatrizoate and sodium diatrizoate (Schering AG). This mixture is then emulsified in Lipiodol. Urografin has a specific gravity of 1.32-1.33, similar to Lipiodol, and a Urografin-Lipiodol emulsion is relatively stable. It is also possible that drugs such as anthracyclines form ion pairs with diatrizoate, and these are partially soluble in Lipiodol (e.g. Epirubicin hydrochloride + sodium meglumine diatrizoate = Epirubicin diatrizoate + sodium chloride + meglumine hydrochloride) (82). Using this technique, it is possible to achieve mixtures that will not separate

even on standing for over 24 hours (83). A pharmacokinetic study of the biodistribution of doxorubicin administered via the hepatic artery has shown that association with Lipiodol lowers the peak blood levels of the drug and increases its intratumoral concentration and half-life, and that these effects are further enhanced by the addition of gelatin sponge embolisation (84). The problem of dissociation of the active agent from its vehicle is also reduced when Lipiodol is used to deliver targeted radiotherapy. The preparation used is ^{131}I - labelled Lipiodol, in which some of the iodine atoms in Lipiodol are replaced by ^{131}I.

1.4 CONSEQUENCES OF HEPATIC ARTERIAL INJECTION OF LIPIODOL

Biodistribution

Localisation in the liver

Attempts to trace the path of Lipiodol through the body have largely utilised ^{131}I-labelled Lipiodol, as ^{131}I is a high energy gamma emitter easily detectable by scinti-scanning. Raoul et al (85) administered Lipiodol via the hepatic artery into 47 patients (23 with HCC, 14 with metastases in the liver and 10 with normal livers), followed by scintigraphy at 1 and 8 days. Nearly all the activity accumulated in the liver(L) and the lungs (*l*), and of this the major component was localised in the liver [L/(L+*l*) > 75% in all 3 groups]. Systemic levels of radioactivity were negligible. Within the liver, the T/NT ratio, comparing activity in the tumour (T) to activity in the surrounding normal tissue (NT) was 4.3 ± 3.6 in those with HCC, and 2.4 ± 0.7 in those with metastases (Figure 1.3). The half-life of activity within the tumour was over 4.5 days. Madsen et al (86) reported that 70 to 90% of a given dose of activity localises in the liver, and 10 to 20% in the lungs, with activity in the blood never exceeding 0.9% of the administered dose. Clearance of activity from normal liver tissue was found to have a half-life of 4 days, while it took 20 to 25% longer for the activity to clear from tumour tissue. Activity in the lungs was found to have a half-life of 5 days. It has been suggested (87) that in some neoplasms, diferent components of the tumour may clear radioactivity at different rates.
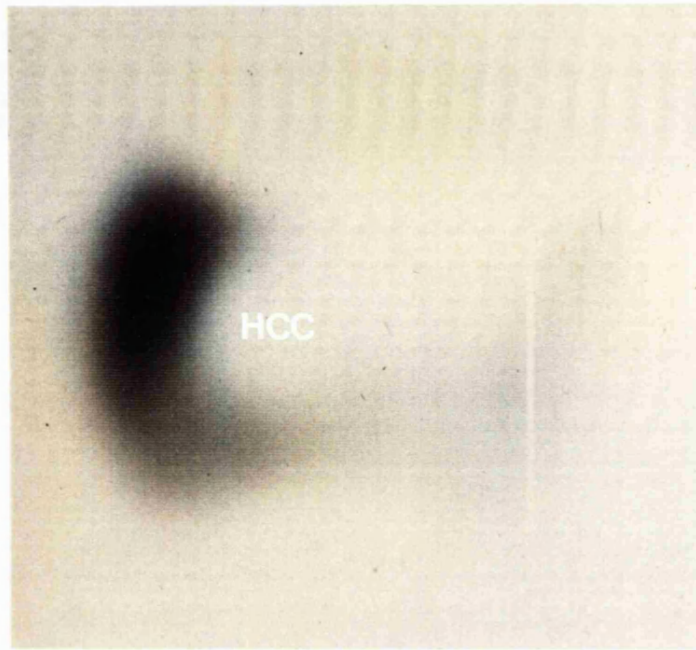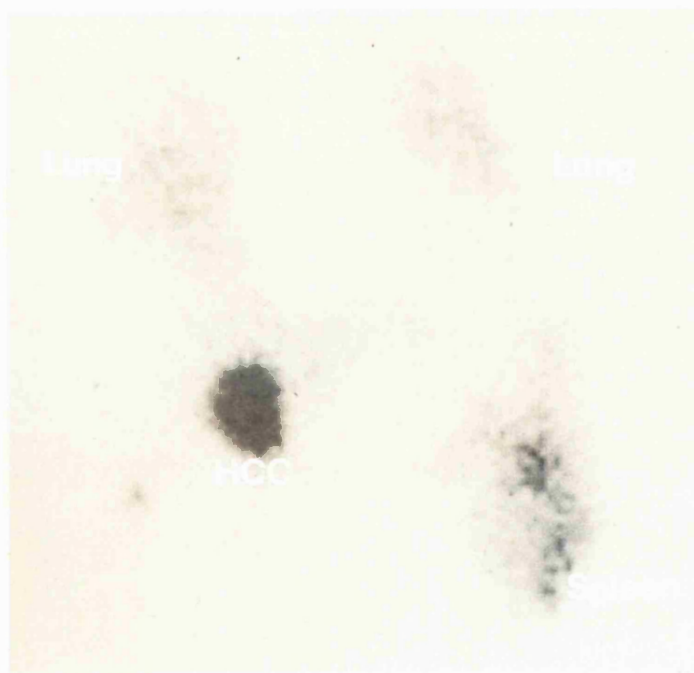
Figure 1.3: Uptake of Lipiodol ^{131}I by a HCC

(A) Technetium colloid scan prior to Lipiodol ^{131}I administration demonstrates the tumour as an area of diminished uptake

(B) Gamma scintiscan 48 hours after Lipiodol ^{131}I administration shows the tumour as a "hot" spot

Despite it being an iodinated compound, there is no significant localisation of Lipiodol in the thyroid, and it has not proved necessary to "block" the thyroid gland by administering iodide preparations prior to Lipiodol injection.

Excretion

Raoul et al stated that 30 to 50% of the activity is excreted in the urine over the 8 days following injection. Very small proportions (<2% over 4 days in a patient with a naso-biliary drain) were excreted via the bile (85). However, when linoleic acid labelled with ^{14}C is administered in a similar fashion, it is primarily excreted in the bile (88). This would suggest that after a period in vivo, the iodine and lipid components of Lipiodol dissociate, the iodine being excreted via the kidneys, while the lipid is excreted into the bile. Dalion's studies on rat livers appear to corroborate this (89).

No changes in biodistribution have been demonstrated on re-injection 4 weeks after the first dose of Lipiodol (85). One may therefore expect repeated courses of Lipiodol-targeted therapy to localise as effectively as on the initial occasion.

Administration of ^{131}I-Lipiodol to patients with liver metastases from colorectal tumours, followed by surgical resection and estimation of activity within the tumour (90) has revealed median T/NT ratios of 1.5:1 at 24 hours and 2.6:1 at 3-9 days, except in very large lesions.

Physiologic consequences and adverse effects

Animal studies

The effects of intra-arterial iodised oil injection on the liver and other organs in a rabbit model were established by Hellekant and Olin (91), using two emulsions with droplet sizes of 15μm and 1 μm respectively. Injection of either into the arterial supply of the liver caused portal pressure to rise by 50%, and a simultaneous fall in cardiac output and arterial blood pressure. Indocyanin green excretion by the liver was also markedly reduced. Hepatic arterial injection had no adverse effects on

the heart, brain or kidneys. Direct injection into the renal arteries did not result in any significant damage to the kidneys, but rapid injection into the coronary arteries and the internal carotid artery had immediate fatal consequences. In humans, accidental embolisation of Lipiodol into the brain following lymphangiography has been associated with neurologic symptoms (92).

Effect on liver haemodynamics

Embolisation of the hepatic artery is possible without compromising the viability of the liver because the portal supply is generally sufficient to sustain the organ. Evidence of a blocked portal vein is accepted as a contraindication for any form of arterially administered embolic therapy. Measurements of the wedged hepatic venous pressure (WHVP) in patients with HCC following hepatic arterial embolisation with gelatin sponge alone (93) have shown a rise in the WHVP (reflecting an elevated hepatic sinusoidal pressure) in 35% of cases. This is probably due to an increase in portal flow to compensate for the reduced arterial input (Pressure = Flow/Resistance). In contrast, injection of gelatin sponge with Lipiodol in a comparable group resulted in a fall in WHVP in 69% of cases. The reason behind this phenomenon remains unclear. Nevertheless, in a clinical situation, the possibility of a rise in portal pressure following embolisation (with or without Lipiodol) should be kept in mind. Gastrointestinal bleeding is known to occur in small proportion of patients following hepatic arterial chemo-embolisation (94, 95). A study using embolisation with starch microspheres (96) has shown that the vascularity of a tumour in relation to its surrounding tissue may be reversed in this manner. This may be used as an adjunct to chemotherapy, whereby flow could be directed preferentially towards tumours that were initially hypovascular.

Microvascular changes

Arterial injection of Lipiodol has been associated with a reduction in the number of vessels manifesting Factor VIII-related antigen in the human liver (97). Microvascular injury has also been demonstrated on scanning electron micrographs of peri-biliary capillary plexuses of the rat liver (98). Lipiodol-targeted therapies may cause a transient rise in
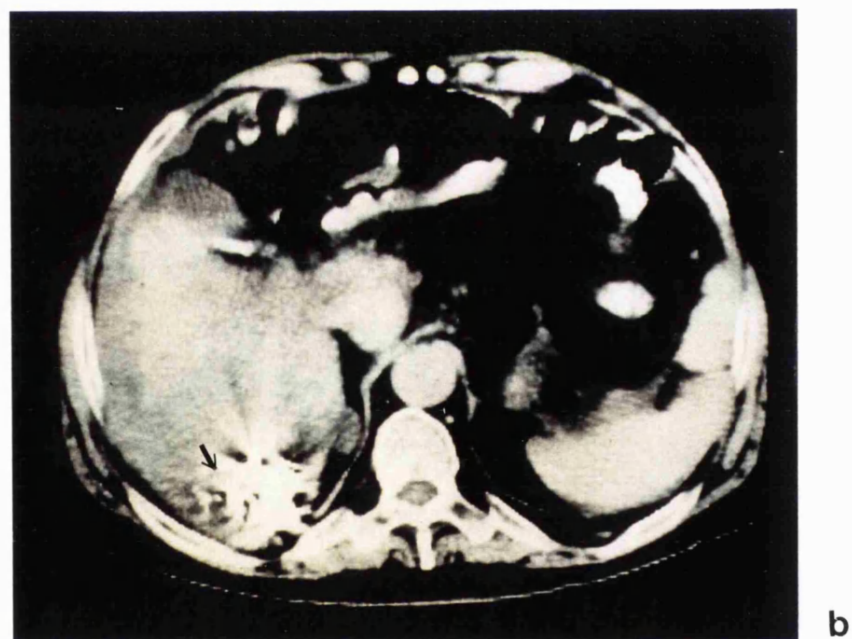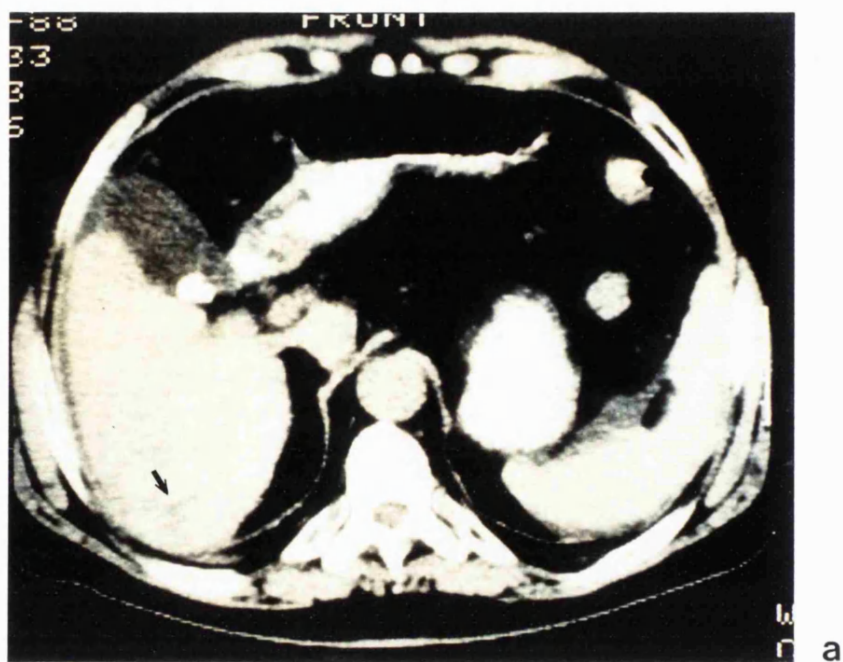
Figure 1.4: CT scan of liver with HCC

(A) Tumour is poorly visualised prior to arterial Lipiodol injection

(B) Following hepatic arterial Lipiodol injection, tumour is visualised as a densely radio-opaque lesion

transaminase enzyme levels (99).

1.5 USE OF LIPIODOL IN THE DIAGNOSTIC IMAGING OF HEPATOCELLULAR CARCINOMA

Lipiodol CT

The imaging modalities commonly utilised in the diagnosis of HCC are ultrasonography, computerised tomography (CT) and hepatic angiography. Ultrasound is a relatively inexpensive and non-invasive test, with a fair degree of sensitivity to justify its use as a screening procedure, though its interpretation is highly observer-dependent. CT scanning is more objective and anatomically easier to interpret. While it has a higher sensitivity, iso-dense tumours and small lesions lying in the "inter-slice" areas may still be missed. Angiography provides an invaluable road-map if surgical resection is being considered, but remains an invasive procedure. A new dimension was added to the investigation of liver tumours by the introduction of "Lipiodol CT" in the mid-1980's, wherein Lipiodol is injected into the hepatic artery at angiography, followed by a CT scan 7 to 14 days later. The retained Lipiodol is radio-dense, and demonstrates the tumour as a high-density area (Figure 1.4).

Sensitivity and specificity

This technique allows detection of primary tumours as small as 2 mm (78, 100). Tiny satellite nodules can also be detected (101), reducing the chances of such lesions being missed at surgery. While routine CT will detect 82% of HCCs less than 5 cm in size, and only 56% of HCCs less than 2cm in size, Lipiodol CT is reported to have a sensitivity of 96% for lesions below 5 cm, and 93% for lesions below 2 cm (102). Erroneous negative interpretations of Lipiodol CT may occur in the case of avascular, necrotic or fibrotic tumours that have not taken up Lipiodol, or if all areas of the liver have not been perfused with Lipiodol. False positive interpretations may be caused by areas of focal nodular hyperplasia, haemangiomas, and metastatic lesions. In such situations, the pattern of Lipiodol uptake within the lesion may give a useful clue:

dense homogeneous opacification is typical of HCC (and may be seen in focal nodular hyperplasia), while a patchy uptake of contrast is suggestive of focal nodular hyperplasia, haemangioma or metastasis. Moreover, areas of focal nodular hyperplasia do not retain Lipiodol for as long as tumours, and follow-up CT scans may be useful in such situations (103).

Quantification of Lipiodol uptake

The degree of Lipiodol uptake in a HCC has been correlated to the extent of histologically demonstrable tumour necrosis (104), and to survival (85). Semi-quantitative criteria for grading Lipiodol retention within a tumour have been proposed by Maki et al (105) (Table 1.5).

Table 1.5

Criteria for grading Lipiodol retention in liver tumours
after Maki et al (105)

Grade 0	No demonstrable Lipiodol uptake
Grade I	Lipiodol retained in less than 10% of the tumour mass (IA), or in less than 10% of the circumference of the tumour (IB)
Grade II	Lipiodol retained in 10 - 50% of the tumour mass (IIA), or in 10-50% of the tumour circumference (IIB)
Grade III	Lipiodol retained in over 50% of the tumour (IIIA), or over 50% of the circumference (IIIB)
Grade IV	Lipiodol retained by the entire tumour mass (IVA), or over the entire circumference of the tumour (IVB)

Lipiodol CT is less useful in the diagnosis of metastatic lesions in the liver, and other modalities such as ultrasonography, CT, and CT during

arterial portography are reported to have a higher sensitivity (106). The use of an ethiodised oil emulsion (EOE 13) as intravenous contrast for CT detection of hepatic metastases has been reported (107). This is taken up by reticuloendothelial cells in the liver (similar to technetium sulphur colloid in isotope scans) and metastatic lesions deficient in reticuloendothelial cells show up as hypodense areas.

1.6   THERAPEUTIC USES OF LIPIODOL

Animal studies

The most commonly used model is the anaplastic VX2 carcinoma (derived originally from a spontaneously transformed papilloma), implanted by direct parenchymal injection into the rabbit liver. Unlike rats and mice, the rabbit has a splanchnic anatomy relatively similar to humans (108). Iwai and colleagues in 1984 demonstrated retention of Lipiodol in these tumours following hepatic arterial injection. Using Lipiodol combined with the lipophilic anti-cancer agent SMANCS, they also demonstrated a significant increase in survival (80). Similar results have been obtained with FdUrd-C8, a lipophilic prodrug of 5-fluoro-2'-deoxyuridine (109). Anti-tumour effects have been demonstrated following intra-arterial administration of Lipiodol-doxorubicin on VX2 carcinoma implanted into the hind legs of rabbits (110).

It has been suggested that lipophilic formulations (as opposed to most cytotoxic formulations available for parenteral use which are hydrophilic) are likely to be more effective when combined with Lipiodol. Konno compared the anti-tumour effects of lipophilic preparations of mitomycin C, aclarubicin and doxorubicin against the effects of lipophobic preparations of the same drugs on the rabbit VX2, and concluded that to achieve the best results, the cytotoxic agent should be soluble in Lipiodol and form a stable combination, with gradual release of the drug into the tumour (111).

Targeted radiotherapy using ^{131}I-Lipiodol has also been successfully demonstrated in HCCs arising in the rat liver, with localisation of activity in the lesions and histologic evidence of tumour necrosis (112).

Targeted chemotherapy for unresectable hepatocellular carcinoma

Cytotoxic agents administered intra-arterially with Lipiodol

Lipiodol alone has no anti-tumour effect (113), and has to be combined with a cytotoxic substance or a radio-isotope to achieve any therapeutic result. Trials of Lipiodol-based chemotherapy have consistently yielded better results (29, 114) than the poor response rates following systemic chemotherapy or intra-arterial chemotherapy using cytotoxic agents without Lipiodol (44). Several cytotoxic agents have been used in conjunction with Lipiodol, including doxorubicin, epidoxorubicin, aclarubicin, 5-fluorouracil, mitomycin, cisplatin and SMANCS. Demonstrated efficacy against HCC, ease of conjugation to the lipid vehicle, and acceptable toxicity are the major considerations that have determined the selection of these cytotoxic agents. Most of them are quite widely used in cancer chemotherapy and a discussion of their pharmacologic properties and adverse effects is beyond the scope of this review. However, some elaboration is required on the use of SMANCS. This is a semisynthetic macromolecular compound, consisting of neocarzinostatin (an antitumour antibiotic protein) and two chains of synthetic copolymers of styrene and maleic acid. It has yet to enter widespread use, but unlike the other drugs, it is lipophilic, dissolves in Lipiodol and forms a stable mixture with it (99). Hepatic arterial administration of Lipiodol alone has not been associated with any significant adverse effects. Thus, apart from the hazards of hepatic artery cannulation, the complications of Lipiodol-based chemotherapy are dependent largely on the systemic effects of the specific cytotoxic agent used. A large proportion of patients suffer some pyrexia, nausea and abdominal discomfort, which is usually self-limiting.

Transcatheter oily chemo-embolisation

Ohishi and colleagues, in 1985, reported on a group of 97 patients who received Lipiodol-based chemotherapy followed by TAE with gelatin sponge. Their results were superior to those obtained by TAE alone on another group of patients at the same centre (78). Takayasu et al in 1987 (113) reported a randomised trial wherein intra-arterial Lipiodol-doxorubicin chemotherapy followed by gelatin sponge embolisation obtained significantly better results than were achieved in a comparable

group with Lipiodol-doxorubicin alone. Subsequently, TAE has been combined with Lipiodol-targeted chemotherapy in several trials, as detailed below. The term commonly used to describe this therapy is chemoembolisation or *transcatheter oily chemoembolisation* (TOCE). The contraindications and potential complications outlined above for TAE, apply to TOCE as well.

Results of Lipiodol-targeted chemotherapy and chemoembolisation: A review

The data from several major trials of Lipiodol-based chemotherapy and TOCE have been summarised in Table 1.6. While a majority of the reports are from the Far East, several studies from Europe and Africa maintain a degree of geographic balance. The literature in this field is growing rapidly, and this review is by no means comprehensive. Several large series have not been included because the treatment regimens, response criteria or survival figures did not allow meaningful comparisons with the other studies. These include a major study from Japan by Konno (99) on the use of SMANCS-Lipiodol in 277 patients, and a report by Bismuth et al (94) on 291 recipients of TOCE with Lipiodol-Doxorubicin and Gelatin sponge wherein a significant number of patients proceeded to receive surgery. Also, in several trials multiple drugs or drug combinations were used and it was not possible to assess the effects of each regimen separately. Results achieved by combining TOCE with with other modalities such as percutaneous ethanol injection (115) have not been included.

It is difficult to draw comparisons amongst these trials. The disease itself is characterised by geographical variations in its natural history, which makes comparisons prone to a degree of fallacy. Most of the reports are retrospective analyses of large consecutive series, and only a few centres have attempted prospective randomised trials. None of the prospective studies, presumably for ethical reasons, has a control group of patients who did not receive any specific treatment at all. Thus, survival benefit conferred by the treatment under consideration may be assessed only by comparison with historical controls from the same centre, or against data from earlier reports on the natural history of HCC (29, 32).

The patient selection criteria in each of the trials have not always been

clearly defined, but by and large, patients with metastatic disease, portal vein occlusion by tumour thrombus, poor hepatic reserve and very poor general condition were excluded.

There is a lack of consensus on the parameters used to judge tumour response and to decide if further courses of treatment are indicated. Most centres have used more than one cycle of treatment, but while some have proceeded to administer multiple doses routinely, others have varied their schedules according to the degree of Lipiodol retention in the lesion or according to the increase or decrease in tumour size and AFP levels. The criteria to determine the number and frequency of cycles need to be standardised. The end points used to judge the relative efficacies of the different regimens also need clarification. Reduction or containment of tumour size and a fall in AFP levels would appear to be acceptable parameters for gauging tumour response in Phase I and II studies, but in the final analysis the duration of survival and the quality of palliation achieved should be the main yardstick to measure one modality against another.

But despite these reservations, it would be reasonable to conclude that Lipiodol-based chemotherapy and TOCE for unresectable HCC can be carried out with an acceptable degree of safety at centres accustomed to treating such patients. Procedure-related morbidity and deaths are well documented, and hepatic failure is a common cause of mortality. Patients with Child C cirrhosis and poor hepatic reserve, advanced tumour and thrombosis of the main trunk of the portal vein are particularly poor candidates for these treatments.

Not all the studies report on the palliative effect, but those that do (116), consistently rate Lipiodol-based chemothrapy and TOCE as providing effective symptomatic relief, and hence, good palliation. ·

The 1-year survivals achieved range from 18% to 81%, and comparisons of relative merit are difficult to make. The addition of Gelatin sponge embolisation to intra-arterial Lipiodol-based chemotherapy does not appear to add to the morbidity or mortality, and may confer a slightly greater survival benefit. That these treatments provide effective loco-regional control but do not affect disseminated disease is borne out by

Table 1.6: Trials of Lipiodol-based chemotherapy/chemo-embolisation for advanced HCC

Authors; Country & Year Treatment used Total patients & their tumour stages	Dosage frequency	Changes in tumour size	AFP levels (if initial titres were high)
Yamashita et al (117) Japan 1992 Lip FUDR 36 patients (35 cirrhotic)	Repeated doses till tumour opacified	Decreased: 24% No change: 57%	Decreased: 68% No change: 32%
Yamashita et al (117) Japan 1992 Lip DOXORUBICIN 67 patients (61 cirrhotic)	Repeated doses till tumour opacified	Decreased: 18% No change: 51%	Decreased: 43% No change: 57%
Kirk et al (118) S. Africa,N. Ireland 1991 Lip DOXORUBICIN 14 patients	1-3 doses at monthly intervals	Decreased: 14% No change: 72%	Decreased: 50% No change: 43%
Kanematsu et al (114) Japan 1989 Lip DOXORUBICIN 149 patients (Okuda I 61/ II 82/ III 6)	Single dose	Decreased: 38%	Decreased: 75%
Leung et al (119) Hong Kong 1992 Lip EPIRUBICIN 30 patients	1-8 cycles (median 3); doses repeated at monthly intervals	Decreased: 7% No change: 20%	Decreased: 16% (by >50% of initial level)
Novell et al (120) U.K. 1991 Lip EPIRUBICIN 35 patients (Okuda I 5, II 23, III 7)	Doses repeated at 2-3 monthly intervals	No change: 43%	Decreased: 9%
Nakamura et al (83) Japan 1989 Lip DOXORUBICIN + Gelatin sponge 100 patients (Okuda I 69/ II 24/ III 7)	Single dose	Not specified	Not specified
Vetter et al (32) France 1991 Lip DOXORUBICIN + Gelatin sponge 30 patients (30 cirrhotic) (Okuda I 8/ II 14/ III 8)	1-5 cycles; doses repeated till tumour progression arrested	Not specified	Decreased: 58% (to normal levels)
Shibata et al (121) Japan 1989 Lip CISPLATIN + Gelatin sponge 71 patients (71 cirrhotic) (TNM I 1/II 16/III 16/IVA 26,B 12)	Mean of 2 cycles per patient (33 received a 2nd dose after one month)	Decreased: 75% No change: 25%	Decreased: 91%
Beppu et al (95) Japan 1991 Lip CISPLATIN + Aclarubicin microspheres 66 patients (AJCC I 1/ II 10/ III 26/ IV 29)	Mean of 3 cycles per patient	Decreased: 77%	Not specified

* depicted graphically but not specified in text

Survival figures	Morbidity	Early post-treatment mortality	Comparisons made and conclusions drawn (by the respective authors)
1 year: 47%	Fever 64%, pain 61%, nausea 39%, liver dysfunction 19%, gastritis, pancreatitis, ascites	None reported	Prospective randomised trial comparing Lip Doxorubicin with Lip FUDR revealed no significant differences in tumour response or patient survival. A
1 year: 28%	Fever 76%, pain 44%, nausea 22%, liver dysfunction 24%, cholecystitis, splenic infarction	None reported	lipophilic ester of FUDR was used.
1 year: 29%	Fever and pain recorded in all patients	7 patients (50%), all with massive tumours, died within 2 months	Despite very advanced stages of disease, a 1 year survival of 28% was achieved, with few local or systemic adverse effects.
1 year: 56% 2 years: 29% 3 years: 17%	Fever 65%, pain 56%, nausea 22%, high AST/ALT 24%, leucopenia 11%	1 death from liver failure	Better results achieved with Lip Doxorubicin in Stages I & II disease compared to arterial ligation/ arterial chemotherapy.
1 year: 18%*	Nausea 80%, alopecia 100%, anemia 50%, leucopenia 33%	3 deaths (10%) in the 1st month	Reduced morbidity but no increase in survival benefit with Lip Epirubicin compared to intravenous Epirubicin.
1 year: 38%	Pyrexia 74%, high AST/ALT 67%, nausea 15%, pain 9% alopecia 7%	3 deaths in the 1st month (2 of liver failure, 1 of marrow suppression)	Offers effective palliation with acceptable morbidity/mortality in patients with Stage I or II lesions.
1 year: 54% 2 years: 33% 3 years: 18%	Transient pain, fever and nausea	3 deaths (3%) in the 1st month (2 from liver failure; 1 of variceal bleed)	Lip Doxorubicin followed by Gelatin sponge yielded better results than embolisation with Doxorubicin/Mitomycin-impregnated Gelatin sponge
1 year: 59% 2 years: 30%	Fever 93%, pain 56%, transient worsening of liver function 100%	3 deaths (10%) in the 1st month	The regimen offered survival benefit (compared to hisorical controls), effective palliation and loco-regional control. Child C cirrhosis had a poor prognosis.
1 year: 55%	Nausea 66%, pain 63%, fever 56%, GI mucosal erosions 24%, jaundice 7% pancreatitis 7%, cholecystitis 3%,encephalopathy 4%	No therapy-related deaths	Results were significantly better than historical controls (n=60) given SMANCS - Lipiodol. Stage IV disease carried a poor prognosis.
1 year: 81% 2 years: 64% 3 years: 51%	Nausea 64%, fever 32%, cholecystitis 4.5%, liver failure 3%, pancreatitis 3%, liver abscess 3%,GI bleed & renal failure 1.5%	No therapy-related deaths	Significantly better survival rate achieved with this regimen, compared to embolisation with Aclarubicin microspheres alone.

the observation in some of the studies that deaths in untreated patients largely occur from hepatic failure, while in those treated the cause of death is usually extrahepatic. A hypervascular tumour that retains large quantities of Lipiodol, disease detected and treated while still in its early stages, and the presence of a good hepatic reserve are indicators of a relatively better prognosis.

Targeted radiotherapy for unresectable hepatocellular carcinoma

Targeted therapy using hepatic arterial injection of microspheres impregnated with ^{90}Y has been attempted with some success. While high levels of activity have been achieved within the tumours by this technique (122), not all studies have reported a decrease in tumour size (122, 123). Leaching of ^{90}Y from the microspheres (causing myelosuppression) and spillage of microspheres into the splanchnic or pulmonary beds (leading to GI bleeds and lung fibrosis) are among the major complications of this therapy (124, 125). The composition and size of the microspheres may have an important role in determining the efficacy and complications (126, 127).

Greater interest has now centred on the use of targeted radiotherapy using ^{131}I-labelled Lipiodol. As mentioned earlier, this localises well in HCCs, and the early reports on its therapeutic use came from France, Japan and South Korea (128-130). Raoul et al (131) have subsequently reported the results of a French multicentre Phase II trial. Fifty patients (47 cirrhotics) with unresectable Okuda Stage I or Stage II HCC were given 1-3 injections of ^{131}I-Lipiodol, with doses ranging from 21-100 mCi. Cumulative radiation dose achieved in the tumour was 6240 ± 5400 cGy. Cumulative doses to nontumorous liver and in the lungs were 550 ± 870 cGy and 290 ± 220 cGy respectively. The treatment was well tolerated. Symptomatic pain relief was achieved in 9 out of 11 patients, and the AFP levels fell in 22 of 29 patients in whom it was initially raised. Decrease in tumour size was seen in 28 of 45 cases (62%), while it remained static in 5 (14%) and increased in size in 11 (24%). Actuarial survival at 1 year was 50% in patients with Okuda Stage I lesions and 19% for Stage II. Patients with poor uptake of Lipiodol on earlier scans and those with Stage III disease, extrahepatic spread and hepatofugal portal flow were excluded from this trial. Yoo et al (132)

have achieved impressive responses with this therapy, with reduction in tumour size in 89% of patients with tumours less than 4 cm in diameter. Both studies have reported no radiation-related complications involving the thyroid, lungs or bone marow.

Pre-operative chemo-embolisation as an adjunct to surgery for hepatocellular carcinoma

The role of pre-operative chemo-embolisation for resectable HCCs remains controversial. There is little doubt that at least partial tumour necrosis is achieved in the majority of patients. Large tumours initially deemed unresectable due to their size may be effectively shrunk by repeated sessions of chemo-embolisation and converted to resectable lesions, as has recently been reported by Yu et al in a series of 30 patients (133). While it may diminish the vascularity of the tumour, troublesome intra-abdominal complications at surgery have been reported following chemo-embolisation, including adhesions, gallbladder infarction, focal liver infarcts and difficulty in identification of the tumour (70). Others have concluded that these changes, if present, do not significantly complicate the operation (133). The extent of these changes may depend on the timing of the embolisation in relation to subsequent surgery (134).

Pre-operative chemo-embolisation has not been consistently associated with diminished recurrence rates or improved survival (114, 135, 136). Adachi et al reported a 3-year follow up on 72 patients with resected HCCs, wherein no improvement in disease-free survival was noted in the 46 recipients of chemo-embolisation (135). Moreover, patients with partial necrosis had a significantly worse survival rate than those with total necrosis or no necrosis, leading to the suggestion that in a partially destroyed tumour the residual cells are more likely to be dislodged into the bloodstream during surgical manipulation. Nagasue et al reported a significant improvement in disease-free survival with Lipiodol-targeted intra-arterial chemotherapy prior to curative resection, but paradoxically in the same study the overall 5-year survival was significantly better in those who did not receive chemotherapy (136). The use of chemo-embolisation prior to transplantation for HCC has been reported (137), but long-term results are awaited.

Post-operative hepatic arterial infusion of Lipiodol-based chemotherapeutic agents following hepatectomy for HCC has been reported to increase the period of disease-free survival, but the cumulative survival rates remained unchanged (138).

Targeted therapy for tumours other than hepatocellular carcinoma

The use of Lipiodol-based hepatic arterial chemotherapy for the fibrolamellar variant of HCC (139) and unresectable hepatoblastoma in infants (140) has been described. Cavernous haemangiomas are known to take up radio-labelled Lipiodol (141), and Lipiodol - Gelatin sponge embolisation may be used as a primary measure for ruptured liver haemangioma (142). Its use in the treatment of secondary tumours in the liver (from gastrointestinal and other primaries) with some success has been reported by Kobayashi et al (143) and Konno et al (144). The effective use of Lipiodol-based local intra-arterial therapies has been reported for primary renal cell carcinoma (145), recurrent breast carcinoma (146) and invasive bladder carcinoma (147).

LIPIODOL INCORPORATION BY LIVER TUMOUR CELLS AND ENDOTHELIAL CELLS IN CULTURE: DEMONSTRATION AND QUANTITATION OF UPTAKE

2.1   BACKGROUND

    Postulated mechanisms of Lipiodol retention

        The microvasculature

        The extra-cellular space

        The reticulo-endothelial system

    The role of tumour cells and endothelial cells: A hypothesis for this study

    Rationale for cell culture studies and choice of cell lines

        Why cell culture?

        The Hep G2 (liver tumour) cell line

        Human umbilical vein endothelial cells (HUVECs)

2.2   MATERIALS AND METHODS

    Cell culture methodology

        Culture of Hep G2 (liver tumour) cells

        Culture of HUVECs

    Exposure of cell monolayers to Lipiodol

        Physical considerations

        Concentrations of Lipiodol used

        Use of tissue culture chamber slides

    Staining of cell monolayers with Oil Red O

        Drawbacks of the Oil Red O stain

    Staining of cell monolayers with Silver Nitrate: Demonstration of Lipiodol incorporation

        Selective nature of Silver Nitrate impregnation stain

        Staining technique

        Demonstration of Lipiodol incorporation

    Quantitation of Lipiodol incorporation: Image analysis

2.3   RESULTS

    Evidence of Lipiodol incorporation by tumour and endothelial cells

        Light microscopy

        Electron microscopy

    Quantitation of the cellular incorporation of Lipiodol by Image Analysis

2.4   CONCLUSIONS

2.1   BACKGROUND

Postulated mechanisms of Lipiodol retention

A substance retained by a HCC following injection into the hepatic artery may lodge in one (or more) of four tissue compartments: (1) the lumina of the tumour blood vessels, (2) the extracellular space, (3) inside tumour cells, endothelial cells (and possibly Ito cells) lining the tumour vessels and (4) inside reticulo-endothelial cells (though typical Kupffer cells may be absent, cells of a phagocytic nature are often present in tumours). There is evidence to suggest that more than one of these tumour components may be involved in the retention of Lipiodol.

The microvasculature

The morphological pattern of Lipiodol uptake within the tumour generally reflects the vascular distribution (143, 148). In small secondary tumours, and in HCCs less than 5 cm in size, there is generally a uniform uptake, while in larger tumours, both primary and metastatic, there is peripheral concentration with a central defect (149). Also, silver nitrate staining of HCCs after Lipiodol angiography indicates that the Lipiodol droplets are lodged mainly in the sinusoids (149).

Several mechanisms have been postulated for how Lipiodol may be retained within the sinusoidal lumen. An altered electrostatic charge on endothelial cells may cause the lipid to adsorb onto the endothelial surface (143). It is possible that the Lipiodol gets into the tumour preferentially due to its increased blood supply, but then cannot exit as the drainage channels from the sinusoids to the veins have been altered (78, 150). It has also been suggested that it simply embolises in the vessels, and that droplet size may determine this effect. If the particles are less than 1 μm in diameter, the lipid is taken up homogeneously throughout the tumour, while particles larger than 15 μm lead to a patchy uptake (91). However, no embolic effect of Lipiodol has been

demonstrated in ultrastuctural studies (149, 150).

The extracellular space

Iwai et al have demonstrated Lipiodol in the extracellular spaces of a tumour in an animal model (88). In normal liver, the sinusoidal wall is characterised by fenestrations and the absence of a basement membrane, which permits particles less than 0.1 μm to pass through. Tumours have similar sinusoids (151), and it may be possible for the lipid to pass through such walls. Moreover, tumour vessels in experimental models are more "leaky" than normal vessels (152, 153). This may be due to an increase in endothelial surface area following tumour angiogenesis (which then provides an increased surface area for lipid transport), the release of permeability-enhancing factors by tumour cells (154), the lack of neural control over the new vessels (155), or due to a decreased rate of blood flow at normal pressure (156). The absence of lymphatics in a liver tumour has also been suggested as a cause for retention of Lipiodol in its extracellular spaces (88).

Reticulo-endothelial system

Evidence for the uptake of oily contrast media by Kupffer cells comes from the studies of Ivancev et al (157, 158), wherein an intravenously administered ethiodised oil emulsion with a droplet size of 1-4 μm was demonstrated by subsequent electron microscopy to have been taken up by Kupffer cells in the liver. Uptake by the reticulo-endothelial system may be the route by which Lipiodol is cleared when administered systemically, as evidenced by its biodistribution to the spleen, bone marrow and lungs (88, 107, 148). Konno et al (144) have postulated that it is the very absence of a reticulo-endothelial component in liver tumours which leads to an inability to take up and clear Lipiodol from the sinusoids. Ultrastructural studies suggest that while Kupffer cells may be present in some compact type HCCs, they are absent in areas of tumour that demonstrate pseudoglandular or trabecular cell patterns (159).

The role of tumour cells and endothelial cells: A hypothesis for this study

With interest largely concentrated on the roles of the microvasculature and the reticulo-endothelial system, there have been relatively few investigations into the role of the tumour cells themselves in the retention of Lipiodol. Ivancev et al (157, 158) in their studies using an ethiodised oil emulsion (EOE-13), demonstrated that droplets smaller than 750 Å in size lodge not in the Kupffer cells but in the hepatocytes. Uptake of lipids is a normal feature of hepatocytes, and in liver tumour cells similar mechanisms are probably at work (85, 148, 157, 158). In several animal models, the major route of excretion of Lipiodol from the liver is via the bile (80, 88, 160). However, a typical histologic characteristic of HCCs is an absence of biliary canaliculi within the tumour architecture (23). Thus, uptake of Lipiodol by tumour cells coupled with an inability to excrete it into the bile may be the reason for prolonged retention of the oil within the tumour.

The endothelial cells lining the tumour vascular spaces in HCCs are known to undergo structural alterations. They may show a multi-layered arrangement, with loss of endothelial fenestrae. Endocytotic vesicles can be recognised in these endothelial cells (159). Also located in the tissue space bordering the sinusoids are atypical cells containing lysosomes, and altered fat-storing (Ito) cells (159, 161). These cells may have an active role to play in the retention of Lipiodol by the tumour.

It is also a well-documented fact that tumours in organs other than the liver take up Lipiodol. Membrane changes involving a decrease in the saturation index (i.e. the ratio of saturated to unsaturated fatty acids) have been demonstrated in a variety of tumour cells (162-164), and Lipiodol uptake may occur as a consequence of these membrane changes.

Thus, Lipiodol retention in tumours may be a consequence of altered cell behaviour following malignant transformation. The experiments described herein are an attempt to study the interaction of Lipiodol with liver tumour cells and endothelial cells in *in vitro* and *in vivo* situations. The *in vitro* (cell culture) studies have been described through chapters 2 and 3; the *in vivo* studies have been outlined in chapter 4.

Rationale for cell culture studies and choice of cell lines

Why cell culture?

In vitro cell culture systems are being increasingly recognised as useful models for toxicity testing of chemicals. In vitro assays have been used for screening of potential anti-cancer agents since 1946 starting with nitrogen mustard, and the correlation of in vitro and in vivo activities of these agents (165) has validated this as a method for toxicity testing. In vivo animal experiments have inherent limitations in analysing certain aspects of biologic processes, which may be more easily studied in in vitro systems. Morever cell culture studies are often less expensive and can be carried out rapidly using smaller quantities of reagents. Unlike animal experimentation, they avoid certain ethical considerations.

Cell culture systems do have several drawbacks (166). It is impossible to replicate in the laboratory the multiplicity of factors at play in vivo. Also, in most cell culture toxicity assays, only the acute toxic effects are measured; long term toxic effects may be gauged only over a period of time in animal models. Tumours are known to demonstrate heterogeneity of behaviour, whereby the effect of a substance on tumour cells in culture may have little correlation to its effect in a clinical setting.

While the effects of water-soluble angiographic contrast media on human endothelial cells in culture have been investigated (167), at the time of the commencement of this project, no similar studies had been reported on Lipiodol in relation to the different cell types within a tumour. Since then, a report of a preliminary nature from South Korea (168) has described Lipiodol uptake in HCC cells in culture. No studies have been reported so far that have quantitatively assessed Lipiodol uptake in different cell lines, and gauged the effects of Lipiodol uptake on cells. In this study, these questions have been investigated by cultivating the relevant cell populations (i.e. endothelium and HCC cells) separately in cell culture, and then assessing their response to the addition of Lipiodol to their environment in concentrations akin to what might be reached in a liver sinusoid.

The Hep G2 (liver tumour) cell line

Of the several human liver tumour cell lines currently available for experimental purposes, the Hep G2 cell line (European Collection of Animal Cell Cultures: Number 85011430) became available in 1979, and since then has been used in over 250 studies. It was derived originally from biopsies of a primary human liver tumour that were overlaid on feeder layers of irradiated mouse cells, and after several months of passage became a feeder-independent proliferating cell line. The tumour of origin was described as a Hepatoma, with features of a Hepatoblastoma as well as a HCC. Hep G2 has a major safety advantage for laboratory experimentation, in that it is free of the Hepatitis B virus, unlike other cell lines such as the PLC/PRF/5 and Hep 3B. It secretes liver-specific proteins, including albumin and alpha-fetoprotein. Hep G2 monolayers express LDL receptors and internalise LDL, VLDL, HDL and chylomicrons. They also take up, secrete and esterify cholesterol, and metabolise it to bile acids (169).

Human umbilical vein endothelial cells (HUVECs)

Primary cultures of endothelial cells that line the tumour vessels in a HCC would have been the endothelial cell line of choice for this study. However, the technical difficulties involved in isolating cellular subpopulations from a specimen of human liver tumour (170) are further compounded by the infrequent availability of sufficient viable human HCC tissue.

HUVECs were first cultured successfully in the early 1970's (171), and are currently one of the most common endothelial cell lines to be grown in culture. Their embryological origin from the umbilical vein is a feature they have in common with hepatic sinusoidal endothelium (172). Also, they represent a rapidly proliferating and not fully differentiated endothelial cell population. Finally, they share several phenotypic markers in common with the tumour endothelium in HCCs, namely Ulex Europeus Lectin, Factor VIII and QBend10 (173).

2.2 MATERIALS AND METHODS

Cell culture methodology

Culture of Hep G2 (liver tumour) cells

The Hep G2 cells used in these experiments were purchased directly from the European Collection of Animal Cell Cultures (ECACC) at the Public Health Laboratories, Porton Down, Salisbury. The cells were grown in 25 ml plastic tissue culture flasks with canted necks. When seeded into a flask, the cells tend to form an adherent monolayer on the floor of the flask within 8 hours. The cultures were passaged on reaching confluence (average time to confluence was 5 days). Their growth requirements and the sub-culture techniques used (169) are outlined below.

(A)   Medium: Dulbecco's Modified Eagle's medium (DMEM) with 10% foetal calf serum, L-Glutamine 2 mmol/l, Penicillin 100,000 units/l and Streptomycin 100 mg/l, with pH between 6.9 and 7.2. Change of medium was required every 48 hours.

(B)   Environment: Humidified air with 5% CO_2 in a standard incubator (Leek), at 37°C.

(C)   Sub-culture ("passage") protocol:

1   The medium from the flask was discarded and the cell layer washed with phosphate-buffered saline (PBS) free of calcium and magnesium.

2   A 10% solution of Trypsin in PBS free of calcium and magnesium was freshly prepared. Five mls of this solution was added to the flask (to cover the entire monolayer) and the cells incubated at 37°C for 10 minutes with intermittent inspection to detect cell detachment.

3   Once the majority of cells detached from the surface of the flask, the Trypsin was rapidly neutralised by addition of an equal volume (5 ml) of DMEM containing 10% foetal calf serum (FCS).

4   The cell suspension was transferred into a centrifuge tube and centrifuged at 300 g for 3 minutes.

5   The supernatant was decanted off and the cell pellet resuspended in DMEM.

6   This suspension was seeded into fresh flasks and incubated in conditions as described above.

Culture of HUVECs

(A)   Harvest protocol:

1   Fresh human umbilical cords were collected from the hospital maternity unit and preserved in M199 medium at room temperature for no longer than 24 hours.

2   A 20-25 cm segment of cord was taken and the umbilical vein was cannulated with soft polythene cannulae at both ends and flushed with PBS. Care was taken not to crush the cord with forceps at any point along its length, and leaks from the vein, if any, were closed with a silk suture.

3   The vein was filled with 15-20 mls of 0.1% Collagenase solution, the cannulae at both ends clamped and the vein incubated at 37°C for 5 minutes.

4   Following incubation the Collagenase solution (now containing a suspension of HUVECs detached from the endothelial lining by the action of Collagenase) was collected in a container, and the vein discarded.

5   An equal volume of M199 with 20% newborn calf serum (NBCS) was rapidly added to the cell suspension in Collagenase to deactivate the enzyme.

6   The mixture was centrifuged at 300 g for 5 minutes and the

supernatant was discarded.

7   Endothelial growth supplement (250 µg in 100 µl PBS) was added to the pellet of deposited cells

8   The deposited cells were resuspended in medium and seeded into 25 ml plastic cell culture flasks with canted necks.

Growth requirements of the cells have been outlined below. The HUVECs formed an adherent monolayer on the surface of the flask in 8-12 hours, and reached confluence in 2-3 days (Figure 2.1). This technique for harvest of HUVECs originally described by Jaffe (171), has been validated in previous studies in the University Department of Surgery, Royal Free Hospital (174). Cells cultured by this method have been confirmed as HUVECs on the basis of morphologic criteria (polygonal 35 x 50 µm cells with a "cobblestone" appearance on light microscopy, and presence of Weibel-Palade bodies on electron microscopy), expression of endothelial markers (Factor VIII-related antigen, thrombomodulin and QBend40), and uptake of acetylated low-density lipoprotein.

(B)   Medium: M199 with 25 mmol/l HEPES, 0.9 mg/l L-glutamine, 6.5-7.0 mg/l sodium bicarbonate, 1.5 mg/l heparin, and 20% foetal calf serum; pH adjusted to 7.2. The medium in each flask was changed every 48 hours.

(C)   Environment: Humidified air with 5% CO_2 in a standard incubator (Leek), at 37°C.

(D)   Sub-culture ("passage") protocol:

1   The medium from the flask was discarded and the cell layer washed with phosphate-buffered saline (PBS) free of calcium and magnesium.

2   A 10% solution of Trypsin in PBS free of calcium and magnesium was freshly prepared. Five mls of this solution was added to the flask (to
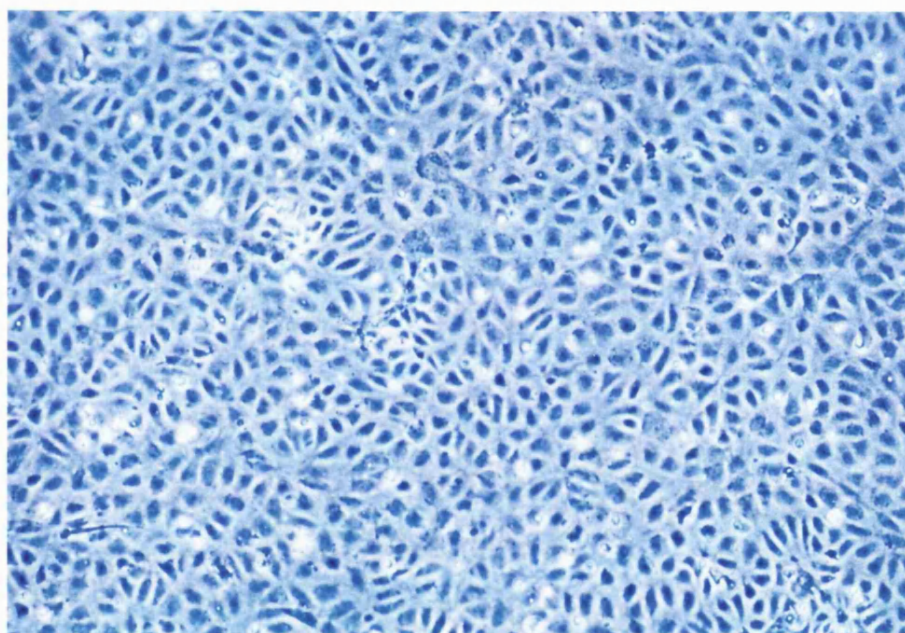
Figure 2.1: Typical "cobblestone" appearance of a monolayer
of human umbilical vein endothelial cells (HUVECs) in culture
(unstained; magnification x 350)

cover the entire monolayer) and the cells incubated at 37ºC for 3 minutes with intermittent inspection to detect cell detachment.

3   Once the majority of cells detached from the surface of the flask, the Trypsin was rapidly neutralised by addition of an equal volume (5 ml) of M199 containing 20% foetal calf serum.

4   The cell suspension was transferred into a centrifuge tube and centrifuged at 300 g for 3 minutes. The supernatant was decanted off.

5   Endothelial growth supplement (250 µg in 100 µl PBS) was added to the pellet of deposited cells.

6   The deposited cells were resuspended in medium and seeded into 25 ml plastic cell culture flasks with canted necks. and the cell pellet resuspended in medium. This suspension was seeded into fresh flasks and incubated in conditions as described above.

Exposure of cell monolayers (Hep G2 and HUVECs) to Lipiodol

Physical considerations

Lipiodol is immiscible with water, and when it is added to an aqueous culture medium such as DMEM or M199, the mixture consists of two distinct phases (Figure 2.2 A); emulsification of Lipiodol can be achieved only by vigorous agitation for a minimum of two minutes. The sizes of lipid droplets in such emulsions of 1%, 2% and 4% Lipiodol range from 4 to 250 µm, but the overwhelming majority (90 to 98%) are less than 25 µm in diameter. The oil-in-water emulsion thus formed gradually separates if allowed to stand for a period of over 1 hour, but the heavier lipid phase does not coalesce and settles in the form fine droplets, with no significant change in droplet size even after 24 hours. Thus, if such an emulsion is added to a monolayer cell culture, after a while multiple small droplets of lipid settle over the floor of the well, covering the monolayer (Figure 2.2 B). Histological studies of tumours injected with Lipiodol suggest that even in vivo, Lipiodol comes in contact with tumour cells in a similar fashion, when droplets of oil lodge in the
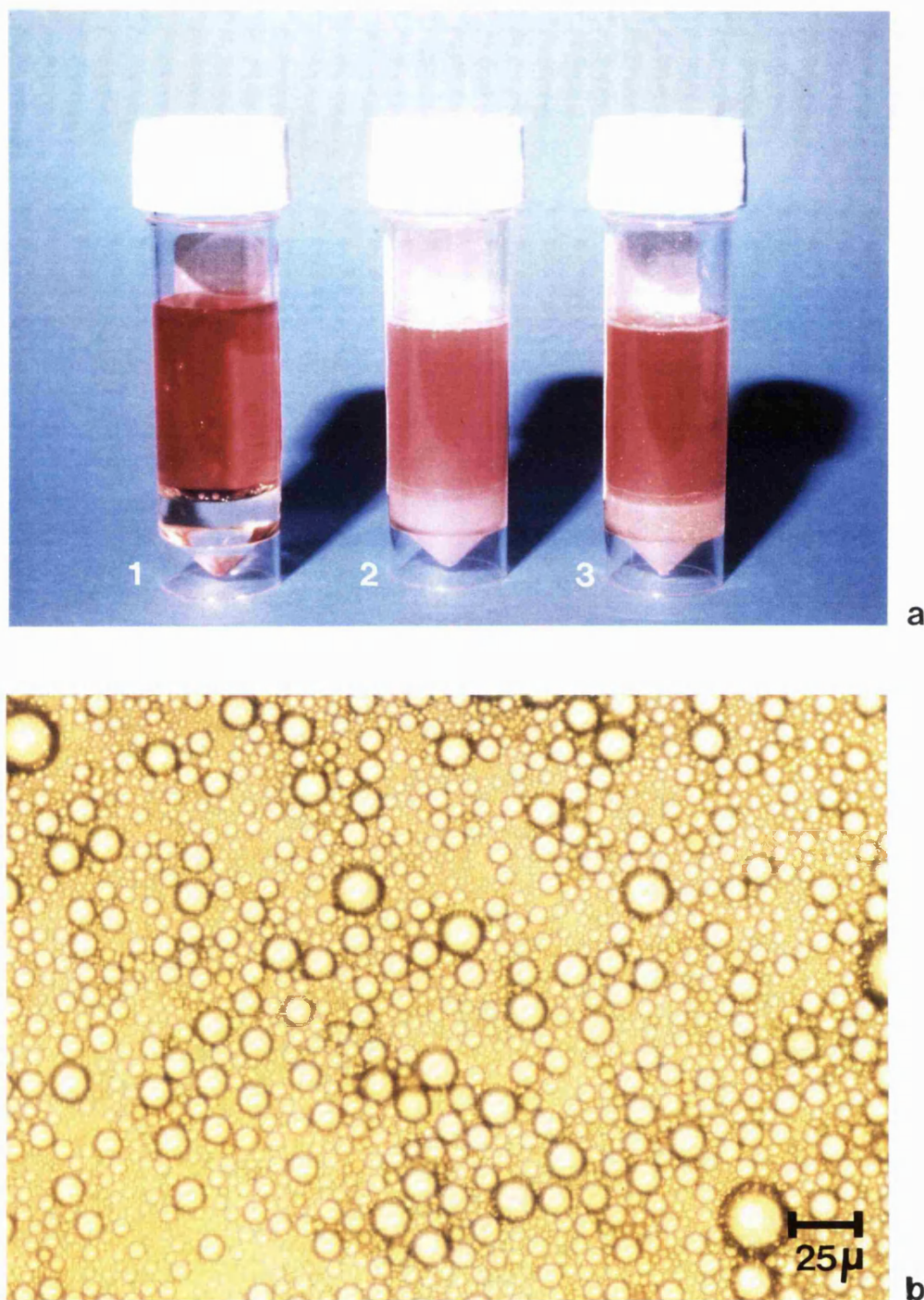
Figure 2.2:   Emulsion of Lipiodol in an aqueous culture medium (DMEM)

(A) On addition of Lipiodol to DMEM (vial 1), the iodised oil settles at the bottom, and the two phases can be clearly distinguished. On agitation for 5 minutes, the oil gets dispersed in the medium in the form of tiny droplets (vial 2). When this emulsion is allowed to settle for a period of 10 minutes (vial 3) the oil droplets settle down again, but do not coalesce. (B) Floor of a tissue culture well to which an emulsion of Lipiodol in DMEM (2% vol/vol) has been added. Multiple droplets of Lipiodol are in apposition to the cell monolayer.

vascular spaces within the tumour.

The use of cell monolayers in cytotoxicity assays is well documented (175), and was adopted in this study as both the cell populations in question (Hep G2 and HUVECs) demonstrate adhesion to plastic and glass surfaces. It has the advantage of requiring low cell numbers. In the case of HUVECs, the cells were used after two to three subcultures to reduce variability amongst replicates.

Concentrations of Lipiodol used

Lipiodol was added to culture medium (DMEM or M199) to achieve concentrations of 1%, 2% and 4% (vol/vol). These concentrations are based on the calculation that average blood flow through the hepatic artery is approximately 500-600 mls/min (2, 176), and injection of 10 ml Lipiodol over a period of 1 minute would lead to a concentration of around 2%.

Use of tissue culture chamber slides

To simplify the task of exposing cell cultures to Lipiodol and then performing histological staining on them, "Lab-Tek" tissue culture chamber slides (Nunc Inc.) were used. These are transparent plastic (Permanox) slides with detachable tissue culture chambers. On completion of the cell culture experiment, the chambers can be detached and the adherent cell monolayer on the surface of the slide can be stained and processed in the routine fashion (Figure 2.3 A and B). However, the slides do not withstand immersion in organic solvents such as xylene, and staining techniques have to be modified accordingly.

Cells (Hep G2 and HUVECs) were seeded into the wells following routine "passage" as described above, with 0.25 ml of cell suspension (containing 1×10^4 cells with a viability of 70-75% as measured by Trypan Blue dye exclusion) added to each well. After overnight incubation (minimum of 8 hours), the cells formed an adherent monolayer on the floor of the well i.e. on the surface of the slide. The medium in each well was then discarded and fresh medium added which contained graded amounts of Lipiodol (Figure 2.3 C).
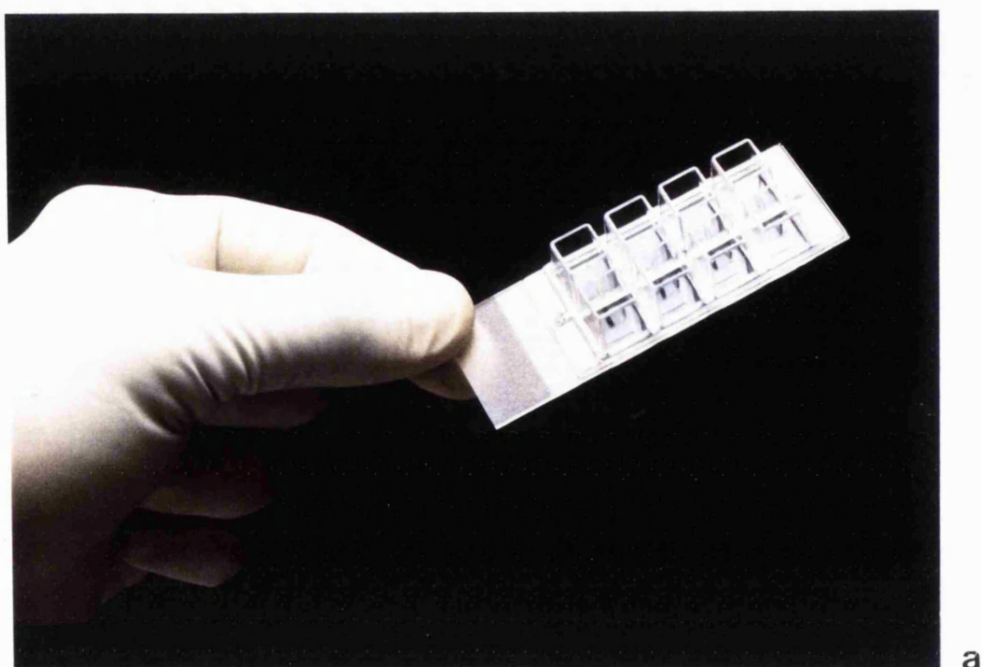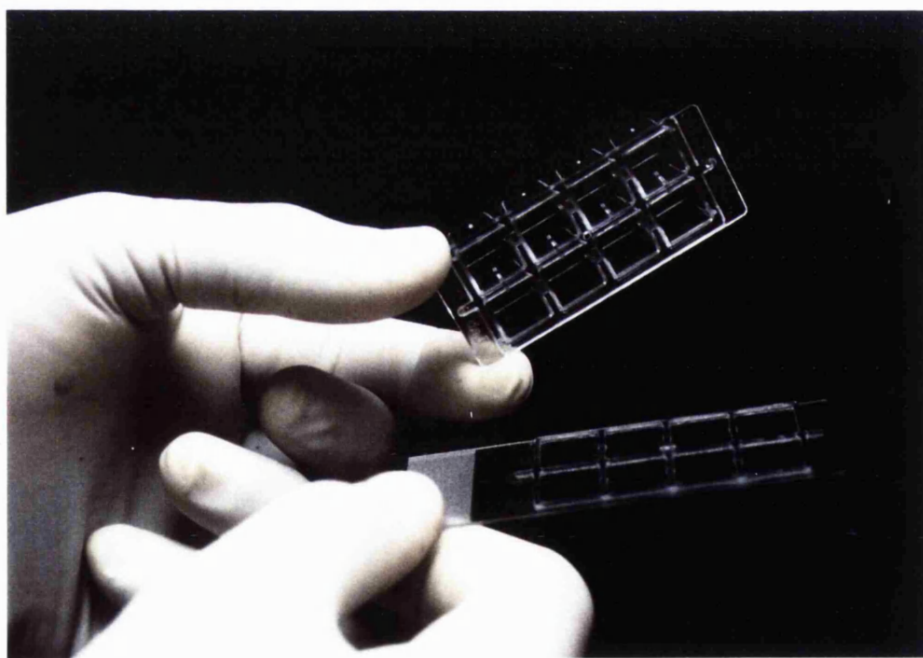
Figure 2.3:   "Lab-Tek" tissue culture chamber slides

(A) Each slide consists of eight tissue culture chambers

(B) On completion of the experiment, the chambers are detached, leaving eight distinct cell monolayers adherent on the surface of the slide, which may then be stained or processed as required for histologic study
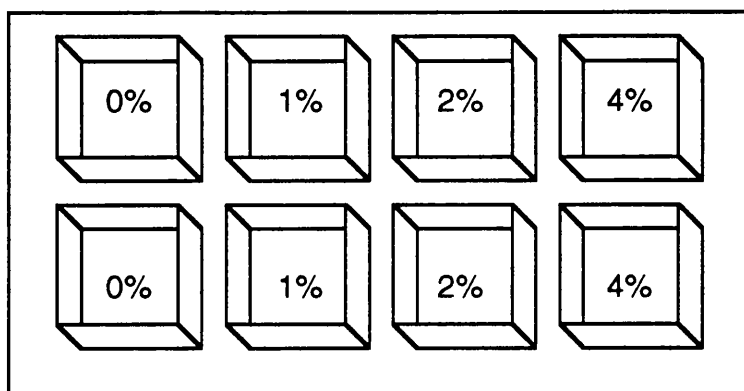
Figure 2.3 (C):   Addition of graded concentrations of Lipiodol
to the culture medium in the 8 cell culture chambers on a "Lab-Tek"
slide (0%, 1%, 2% and 4% vol/vol)

Of the 8 wells on each chamber-slide, 2 received medium without any Lipiodol (controls), 2 received medium with 1% Lipiodol, 2 received medium with 2% Lipiodol, and 2 received medium with 4% Lipiodol (Figure 2.3 C). The chamber slides were then incubated in the usual manner for periods of 8, 12, 24 and 32 hours, and then subjected to staining as described below.

Staining of cell monolayers with Oil Red O

Oil Red O is a commonly used Sudan dye which stains unsaturated hydrophobic lipids and mineral oils *red* and some phospholipids *pink*. It stains Lipiodol *red* (this was confirmed by adding Oil Red O to a slide coated with a thin film of Lipiodol). The staining technique used was adapted from Bancroft and Stevens (177).

Oil Red O staining technique:

1   Oil Red O stock solution (saturated 0.5% solution of Oil Red O in absolute isopropanol) was mixed with distilled water in a proportion of 3 : 2 (vol/vol), allowed to stand for 10 minutes and filtered before use.

2   The cell monolayers were rinsed with 60% isopropanol

3   Oil Red O was added to each well so that the monolayer was immersed in Oil Red O for 10 minutes.

4   The monolayers were rinsed briefly in 60% isopropanol, and washed with distilled water.

5   Mayer's Haemalum was added to each well (to cover the monolayer) and allowed to stand for 1 minute, to counterstain the nuclei.

6   The wells were then detached, leaving the stained cell monolayers on the surface of the slide.

7   The slides were washed in tap water and mounted in glycerine jelly (Dako Glycergel).
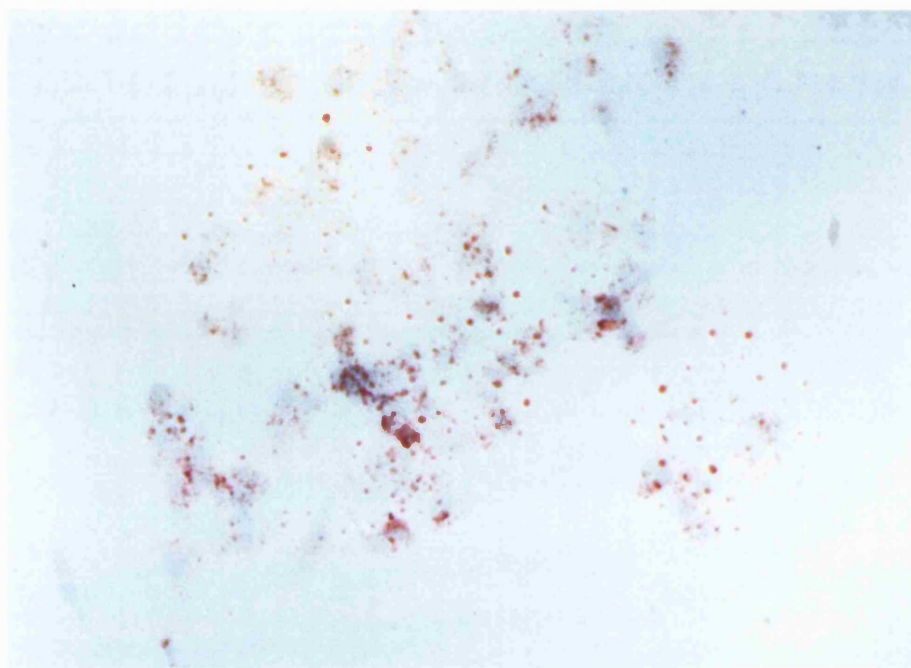
Figure 2.4: Oil Red O stain of HepG2 monolayers exposed to 2% Lipiodol for 8 hours (Counterstain: haematoxylin; magnification x 350)

Drawbacks of the Oil Red O stain

Oil Red O is not a selective stain for Lipiodol. When cell monolayers were stained with Oil Red O after exposure to Lipiodol, numerous red intracellular lipid droplets were visualised, suggestive of Lipiodol incorporation by the cells (Figure 2.4). However, Hep G2 cells and HUVECs normally contain endogenous cytoplasmic lipid vesicles. Even in "control" cell monolayers that had not been exposed to Lipiodol, some endogenous cytoplasmic lipid droplets were stained red by Oil Red O, and it was not possible to distinguish endogenous lipid from incorporated Lipiodol.

Staining of cell monolayers with Silver Nitrate and demonstration of Lipiodol uptake

Selective nature of Silver Nitrate impregnation stain

A histologic method of demonstrating Lipiodol in cryostat sections by impregnating the tissue with silver nitrate was reported by Felton in 1952 (178). The Lipiodol was visualised as a golden brown or black silver iodide precipitate. This method was successfully modified by Arnold et al for tissue paraffin sections (179), and their technique was further modified in this study for use on cell monolayers. Silver nitrate is a *selective* stain for Lipiodol to the exclusion of endogenous intracellular lipids. This was convincingly demonstrated in experiments outlined below, wherein cell monolayers were stained with silver nitrate before and after exposure to Lipiodol. Also, human tissue sections of fatty liver, which clearly demonstrate the lipid accumulation on osmium staining (Figure 2.5 A), do not show comparable staining with the silver nitrate method (Figure 2.5 B). This has been further discussed in chapter 4.
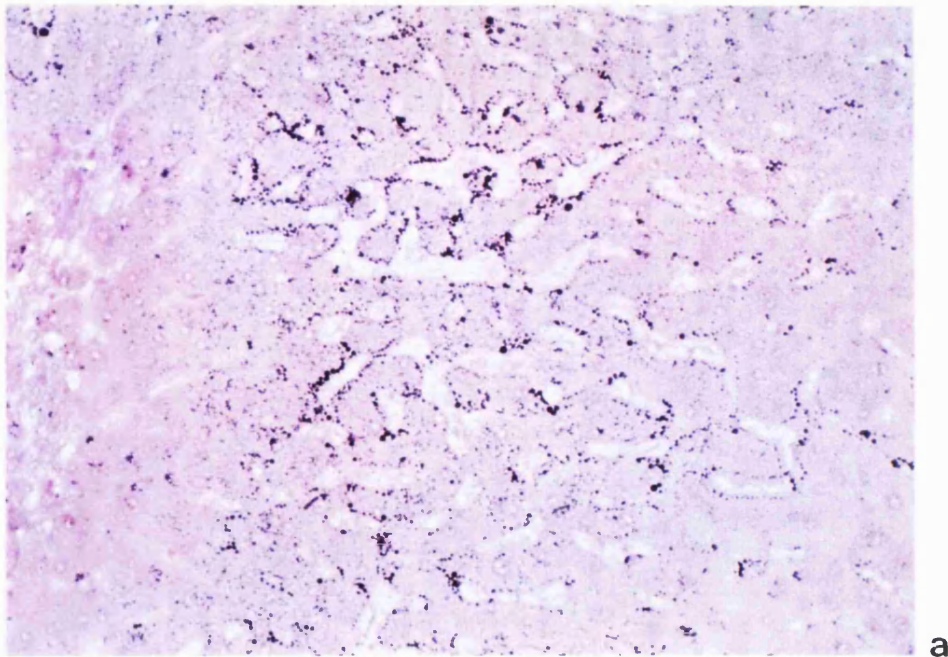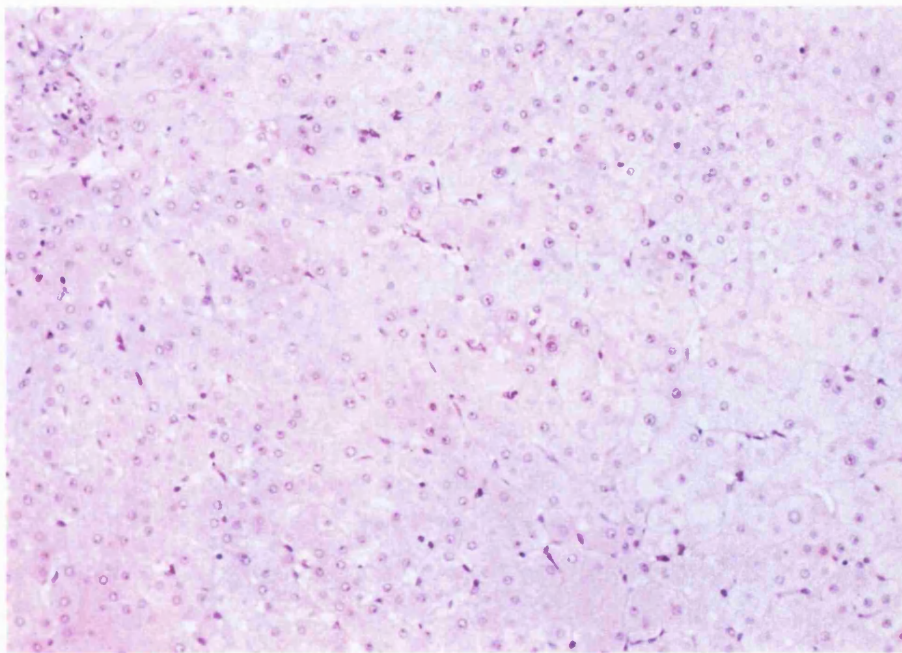
Figure 2.5:   Silver nitrate impregnation does not stain endogenous fat
(A) Demonstration of lipid deposition in a fatty liver with osmium stain (Haematoxylin
and eosin; magnification x 150)
(B) Contiguous section of same liver impregnated with silver nitrate - endogenous fat is
not visualised (Haematoxylin and eosin; magnification x 150)

Staining technique

(A)   Silver Nitrate impregnation

1   After exposure to Lipiodol, formalin (10% formaldehyde in 0.9% saline) was added to the wells and left overnight to achieve histological fixation of the cell monolayers.

2   The formalin was washed off by rinsing in distilled water for 5 minutes, and the cell monolayers were left to soak in distilled water overnight (to remove excess formalin and thereby avoid possible formalin-induced silver precipitate).

3   The cell monolayers were rinsed in 70% ethanol for 1 minute to remove excess Lipiodol adherent to the floor of the wells (other reagents were also used to remove excess Lipiodol, including 100% ethanol and the detergents Triton and Tween 20, but in a series of preliminary validation experiments 70% ethanol gave the best results without significantly reducing the amount of intracellular Lipiodol).

4   Further rinsing was carried out with distilled water to clear the ethanol.

5   Freshly prepared silver nitrate solution (2.5% in distilled water) was added to the wells, and left for 60 minutes at 4°C in the dark.

6   Following this period of exposure, the monolayers were washed with distilled water to remove excess silver nitrate. The specimens could then be stored for upto 7 days at 4°C before any further counter-staining was performed.

(B)   Counter-staining

Counter-staining for cellular morphology was carried out using either Carazzi's Haematoxylin or Neutral Red. The standard methodology described for routine tissue sections was modified wherever necessary to achieve optimum optical results with the cell monolayers.

Carazzi's Haematoxylin is a blue counterstain for nuclei. It requires a

short staining time, and the intensity of staining can be adjusted by using a "progressive" staining technique. Carazzi's haematoxylin solution (haematoxylin 0.5 g, potassium alum 25 g, potassium iodate 0.1 g and glycerol 100 ml, all dissolved in 400 ml distilled water) was added to the wells, and allowed to stand for 4 minutes, before the excess was removed by rinsing in distilled water for 1 minute. The monolayers were then "blued" by rinsing in tap water for 2 minutes.

Counterstaining with Neutral Red was carried out by addition of 1% Neutral Red solution to the wells for 45 seconds and the excess was then washed off with distilled water for 1 minute.

On completion of staining, the wells were detached from the slide and the air dried slides mounted using the clear adhesive mounting medium Loctite™, set by exposure to Ultra-violet light for 5-10 minutes. The tissue culture chamber slides bend and get distorted on exposure to xylene, and the standard mounting techniques could not be used. Other mounting media (Apathy's medium, Dako glycergel) were tried, but Loctite gave the best visual results on light microscopy.

Demonstration of Lipiodol uptake

All cell monolayers exposed to Lipiodol and stained with silver nitrate were studied under the light microscope. Three batches of Hep G2 monolayers, fixed in glutaraldehyde and not stained with silver nitrate, were processed for transmission electron microscopy (the processing of specimens was carried out by Ms J Lewin in the Department of Electron Microscopy, Royal Free Hospital).

Quantitation of Lipiodol uptake: Image Analysis

Colorimetric assays which use colour density as a quantitating yardstick are commonly used to measure cell number, protein content, DNA content, and enzyme activity. Silver nitrate being a selective stain for Lipiodol, the staining intensity of silver nitrate in a cell monolayer can be expected to reflect the extent of Lipiodol accumulation in the cells. A computerised video image analysis system was therefore used to obtain an objective measure of the optical density of staining (180, 181).

The image analysis equipment consisted of: 1) a solid state camera connected to and capturing the image seen through a microscope, 2) a video digitiser with 16-bit RGB colour output and 3) a desktop computer running image analysis software designed specifically for densitometric measurement (Figure 2.6). The software (Chromatic Colour Image Analysis System Version 3.0; Copyright L R Jarvis 1991) allowed the staining optical density and the staining area to be measured in arbitrary standardised units. As correction for uneven illumination, an image of the "white" background was stored before measurement and the pixel values were retrieved and used to correct every subsequent scan. A 550 nm narrow band interference filter and a x 16 objective lens were used for all measurements. The regions of staining were analysed by the computer, according to the relative density of the stain above that of the unstained tissue. As calculated from the area and optical density measurements, *the staining intensity was expressed in arbitrary optical density units for the entire visualised field.* The software also permitted a count of the total number of cell nuclei in the analysed image. Thus, the optical density for the entire field divided by the number of nuclei yielded the *average staining intensity per cell, i.e. a reflection of Lipiodol accumulation in each cell.*

Monolayers of Hep G2 cells and HUVECs in tissue culture chamber slides were exposed for 4, 8, 24 and 32 hours to culture media containing 1%, 2% and 4% of Lipiodol (by volume). For each time period and concentration, 4 specimens (wells) were taken, and from these 4 monolayers, a total of 10 randomly selected fields were subjected to image analysis. For each field, the average integrated optical density per cell was calculated, i.e. a reflection of Lipiodol accumulation in each cell. The arithmetic mean of the 10 values was taken to represent the staining intensity for that time period and concentration (given the variation between repeated readings, n=10 was judged to be an adequate sample size). The data from these experiments have been listed in Appendix 2. Statistical analysis of the data was carried out on an Apple Macintosh computer using the software program 'StatWorks Version 1.2' (© Cricket Software Inc. 1985).
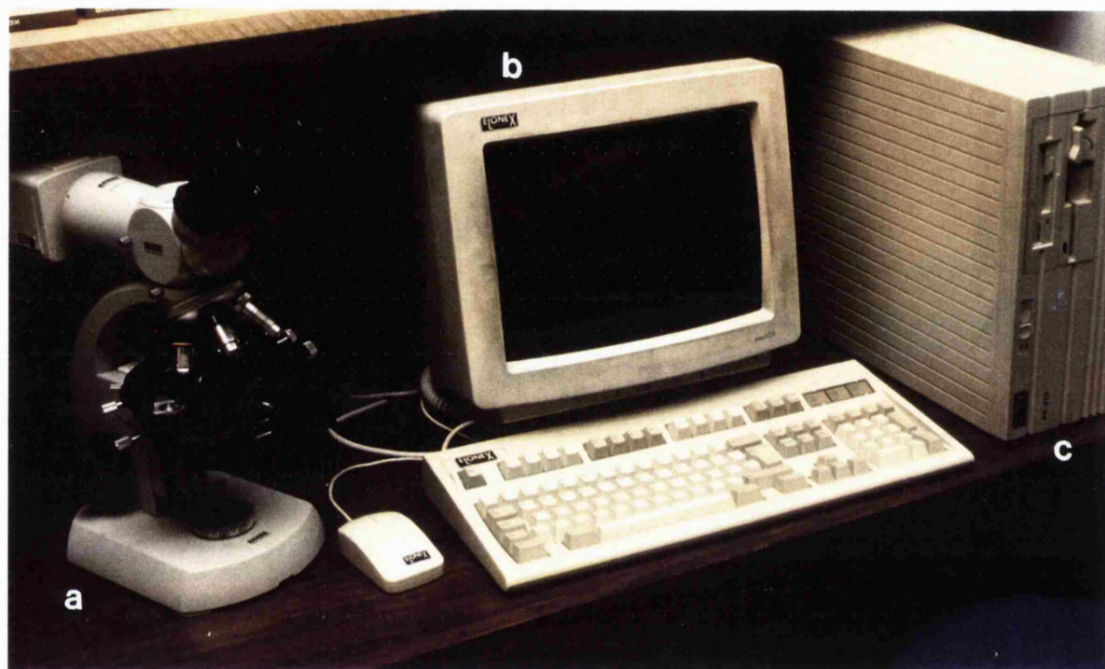
Figure 2.6: Equipment used for computer assisted video image analysis
(a) Microscope with solid state camera (b) video digitiser with colour output
(c) desktop computer

2.3 RESULTS

Evidence of Lipiodol incorporation by tumour and endothelial cells

Light microscopy

Hep G2 monolayers exposed to Lipiodol consistently demonstrated intracellular golden brown vesicles of Lipiodol on silver nitrate staining (Figures 2.7 B, 2.8 B). In cells that had not been exposed to Lipiodol, there were no comparable vesicles (Figures 2.7 A, 2.8 A). Apart from this, no gross visible differences were present between the two groups in terms of cell numbers, appearances of individual cells and their nuclei, and the configuration of cells on the surface of the chamber slide. Similarly in HUVECs, cells exposed to Lipiodol consistently demonstrated intracellular golden brown vesicles of Lipiodol (Figure 2.9), while control monolayers did not. The appearances in both Hep G2 and HUVECs also suggested that cells exposed to Lipiodol for longer periods and at higher concentrations had a higher number of intracellular vesicles, but this aspect was evaluated quantitatively by image analysis (vide infra).

Electron microscopy

Transmission electron microscopy (EM) of Hep G2 cells exposed to Lipiodol revealed unusual multiple intracellular vesicles (Figures 2.10 to 2.13). Given their appearance and their consistent absence in the control cells that had not received Lipiodol, and the findings of light microscopy as stated above, it was concluded that these intracellular vesicles represented incorporated Lipiodol or some derivative thereof. Inspection at high magnification indicated that these were membrane-bound vesicles, suggesting pinocytosis as a likely mechanism of incorporation. Some of the vesicles were seen in association with lysosomes (Figure 2.13). Of interest was the overall healthy appearance of the cells despite the presence of Lipiodol vesicles within their cytoplasm.
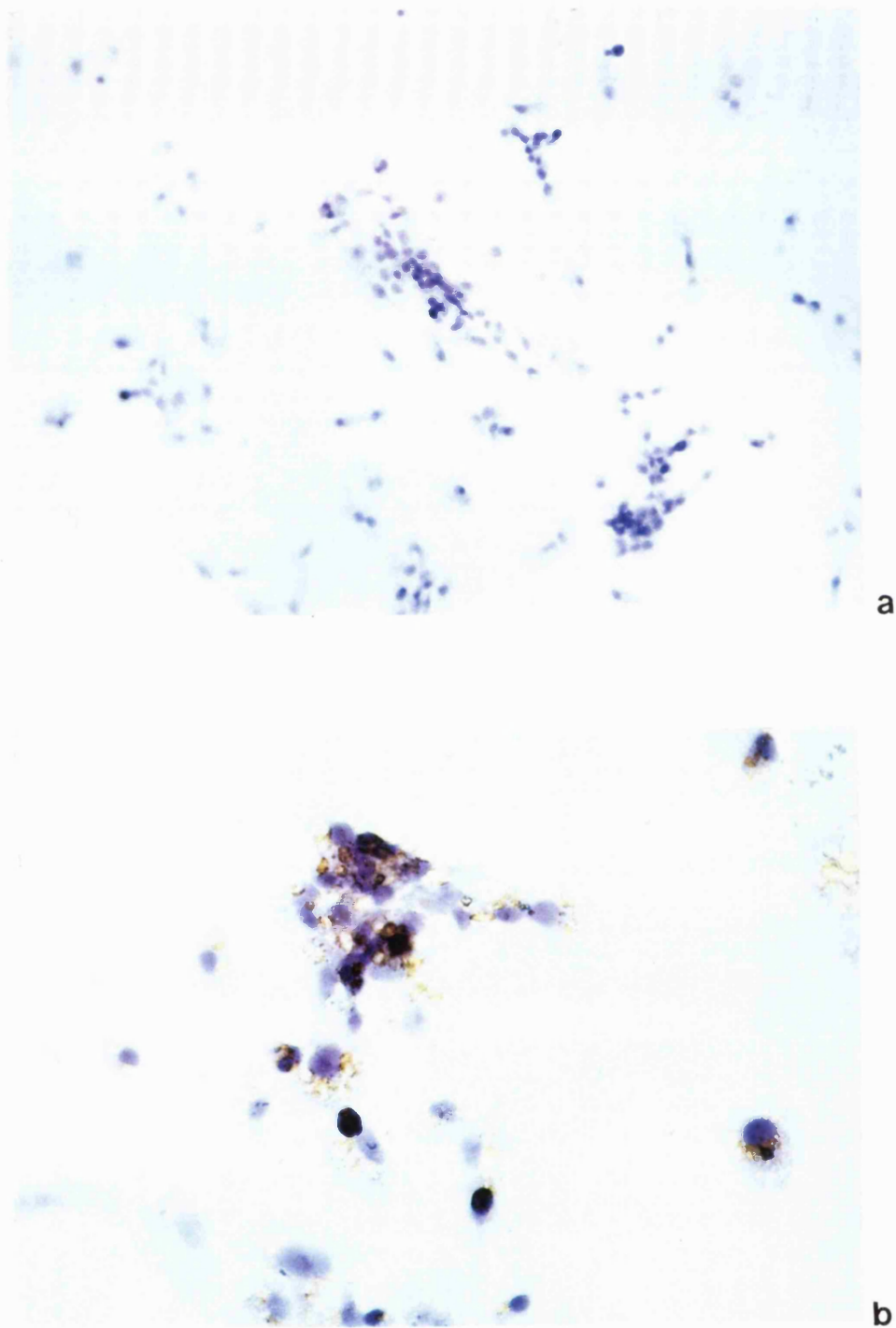
Figure 2.7:   HepG2 cell monolayer impregnated with silver nitrate and counterstained
with haematoxylin
(A) not exposed to Lipiodol (magnification x 200)
(B) exposed to 2% Lipiodol for 8 hours (magnification x 550); Lipiodol visualised as
golden brown intracellular vesicles
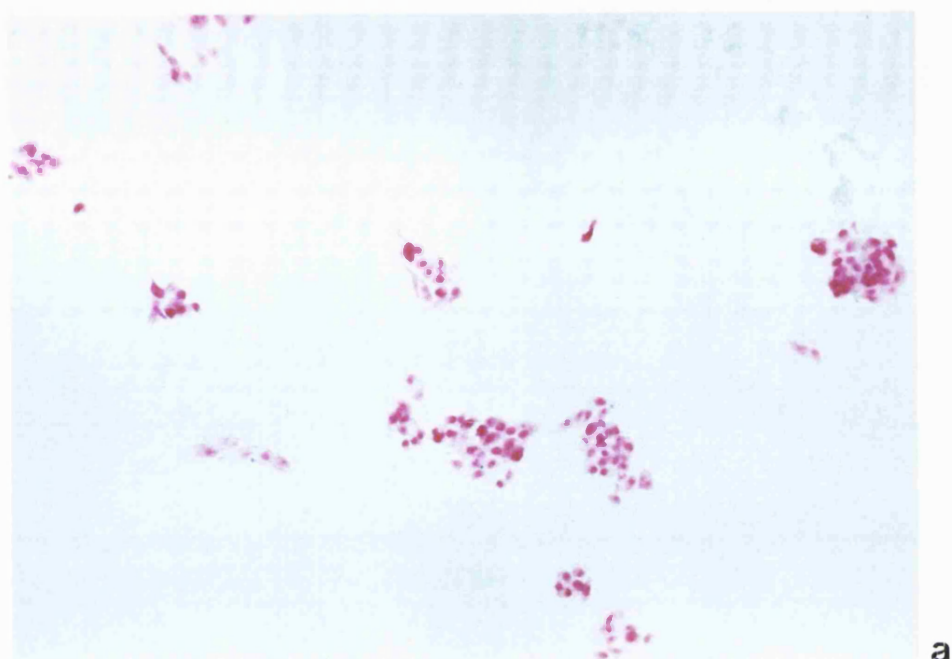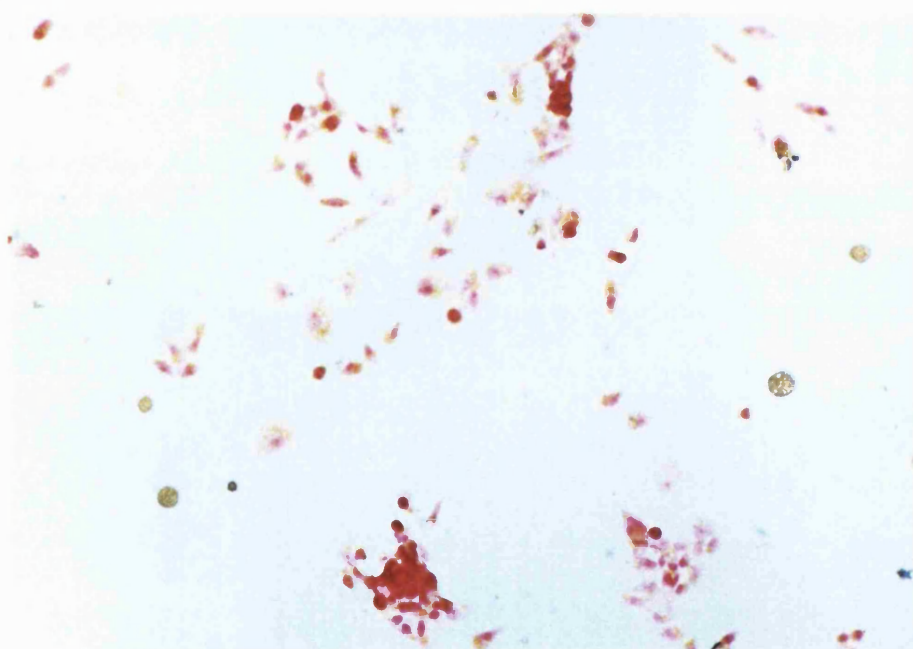
Figure 2.8: HepG2 cell monolayer impregnated with silver nitrate and counterstained with neutral red

(A) not exposed to Lipiodol (magnification x 200)

(B) exposed to 2% Lipiodol for 8 hours (magnification x 250); Lipiodol visualised as golden brown intracellular vesicles

Figure 2.9: HUVECs in culture, prior to attaining confluence, exposed to
2% Lipiodol for 8 hours
(silver nitrate impregnation and haematoxylin counterstain; magnification x 550)
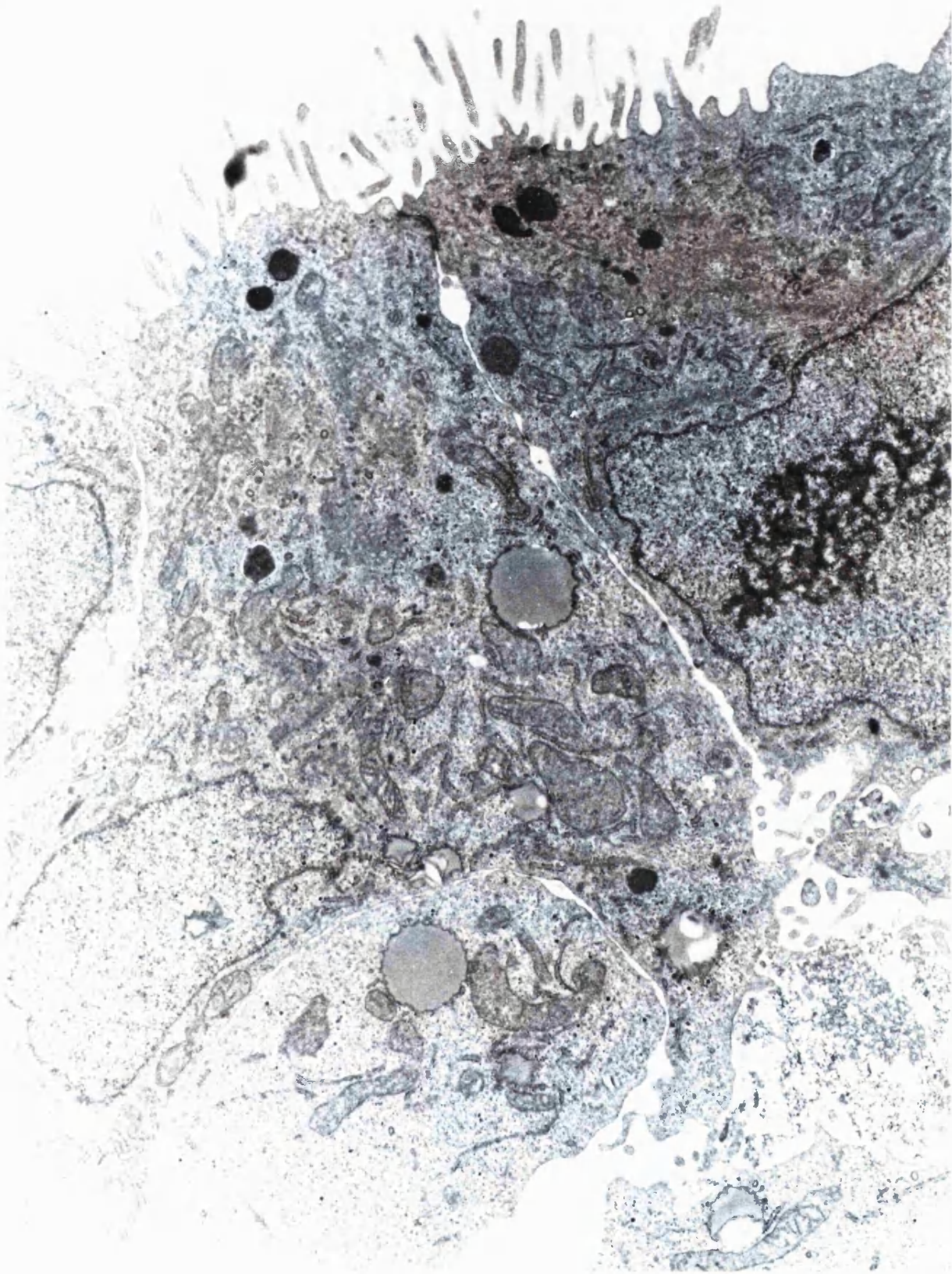
Figure 2.10: Transmission electron micrograph of HepG2 cell monolayers exposed to 1% Lipiodol for 32 hours (magnification x 14900), characterised by multiple cytoplasmic vesicles of varying sizes, with unusual crenated margins, some in association with lysosomes. These were consistently absent in cells that had not been exposed to Lipiodol, and probably represent incorporated Lipiodol or a metabolite thereof.
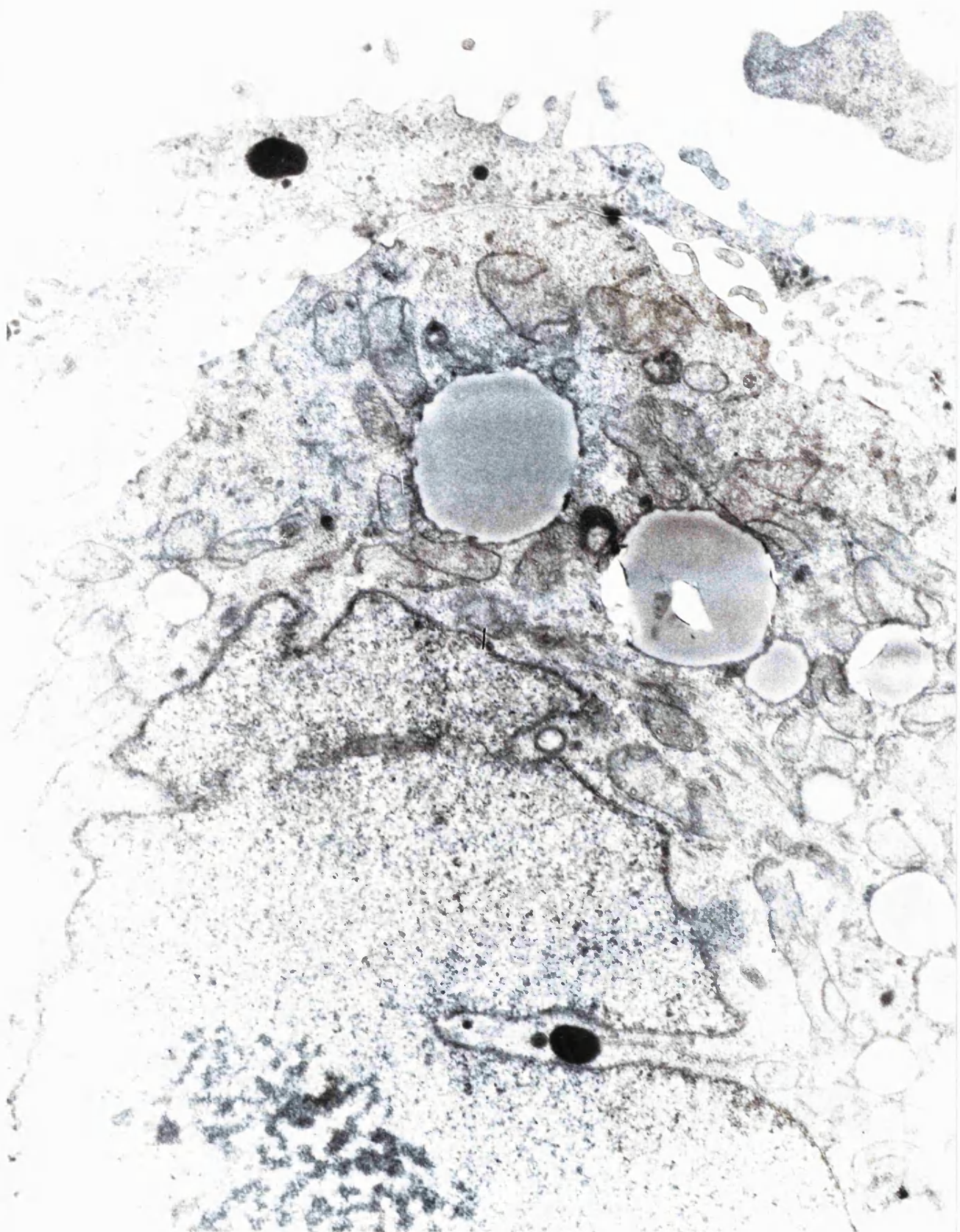
Figure 2.11:   Transmission electron micrograph of HepG2 cell monolayers exposed to
2% Lipiodol for 32 hours (magnification x 22400). Multiple cytoplasmic vesicles of varying
sizes, with unusual crenated margins, probably representative of incorporated Lipiodol or
a metabolite thereof.
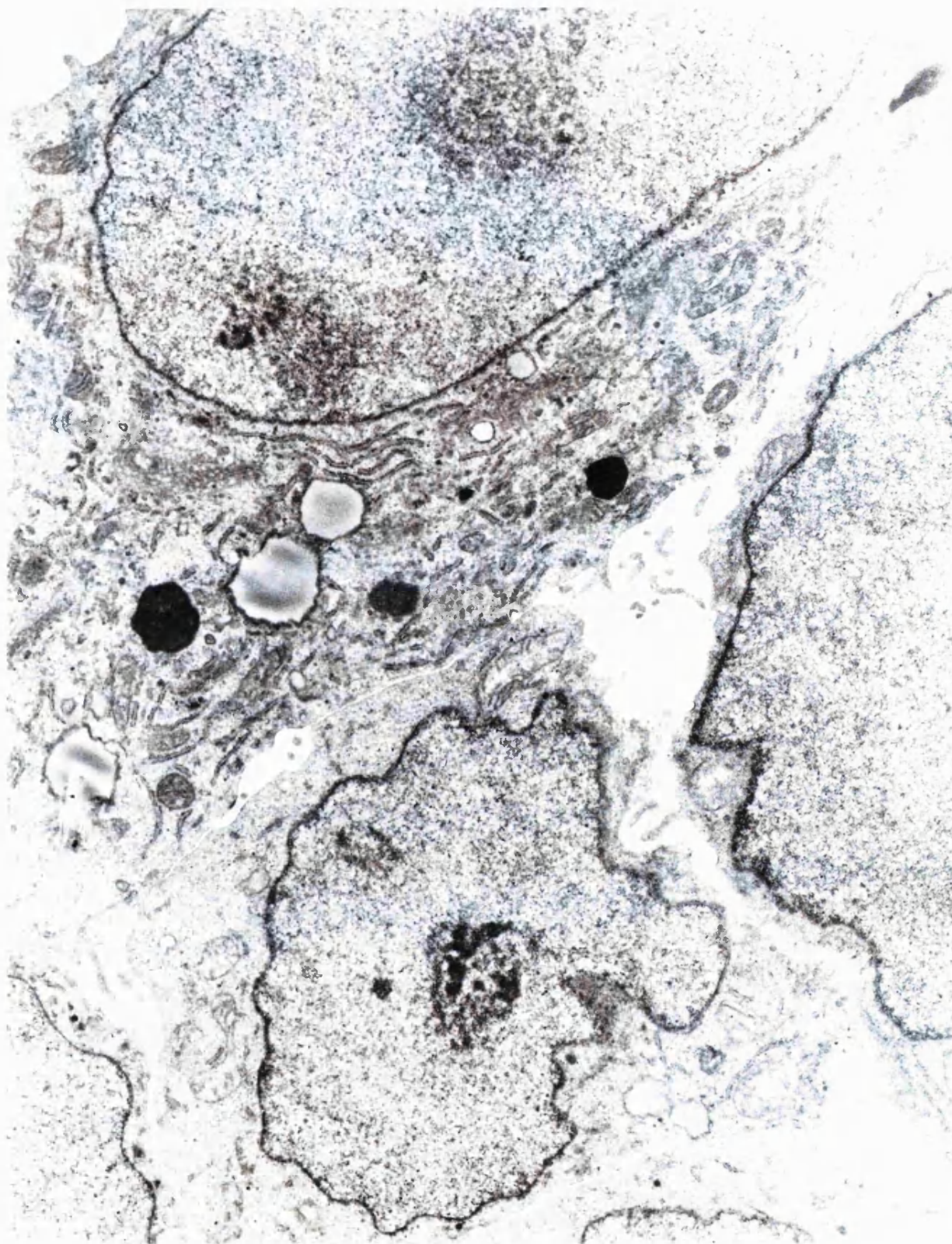
Figure 2.12:   Transmission electron micrograph of HepG2 cell monolayers exposed to 2% Lipiodol for 32 hours (magnification x 14900). Further demonstration of multiple cytoplasmic vesicles, probably representative of incorporated Lipiodol or a metabolite thereof.

Figure 2.13:   Transmission electron micrograph of HepG2 cell monolayers exposed to 2% Lipiodol for 32 hours (magnification x 32000). Examination of the cytoplasmic vesicles at a higher magnification indicates that they are membrane-bound. This would suggest that the Lipiodol was incorporated by a process of pinocytosis.

Quantitation of cellular incorporation of Lipiodol by image analysis

Effect of Lipiodol concentration and duration of exposure on uptake

For both cell types, the optical density of every monolayer exposed to Lipiodol was compared to the optical density of its 'control' monolayer. In every instance a statistically significant increase in optical density was noted following exposure to Lipiodol [$p<0.05$ (values ranged from 0.003 to 0.03); Wilcoxon Signed Rank test for non-parametric data].

The effect of Lipiodol concentration and duration of exposure on the incorporation of Lipiodol by HepG2 is depicted in Figure 2.14. At the lowest concentration (1%) the amount of Lipiodol in the cells - as represented by the optical density - started to increase only after a period of 24 hours, and was still rising at 32 hours when the experiment was terminated. With 2% Lipiodol, the lag period was reduced to 8 hours. At a higher concentration (4%), incorporation of Lipiodol was more rapid and reached a plateau within 8 hours.

Lipiodol incorporation by HUVECs, as shown in Figure 2.15, followed a different pattern. At 4 hours, for all concentrations of Lipiodol, there was a four- to seven-fold increase in optical density compared to the controls, indicating a rapid entry of the oil into the cells. This was followed by a decrease in optical density over the next 4 hours. As experiments outlined in the following chapter demonstrate, this decrease in optical density was *not* due to any decrease in the total number of cells, and probably reflected intracellular events whereby the Lipiodol was either excreted back into the surrounding medium or metabolised by the cell into products that did not stain with silver nitrate. But over the next 16 to 24 hours, there was again an increase in optical density (for all concentrations), followed by a decrease (in 2 out of 3 concentrations). This suggests that given the continuing presence of Lipiodol in the surrounding media, the cells maybe repeating the same process.

Figure 2.14

Results of computerised image analysis:
Effect of Lipiodol concentrations and duration of exposure
on Lipiodol incorporation by HepG2 cells
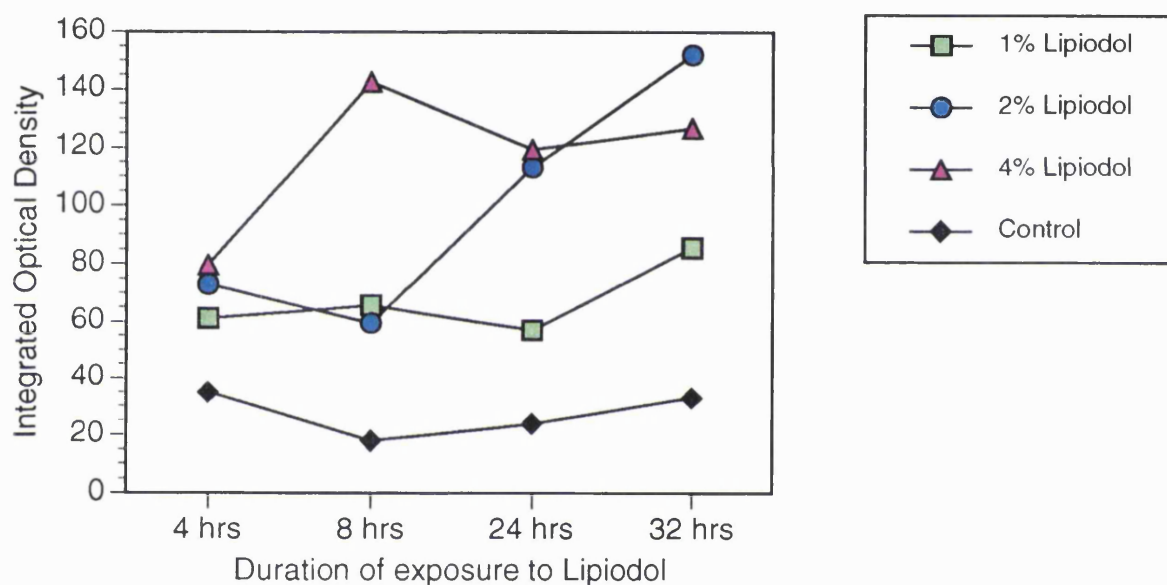
Figures represent average integrated optical density (IOD) per cell in cell monolayers
following exposure to Lipiodol (Silver nitrate stain for Lipiodol, counterstained with
*Carazzi's Haematoxylin); Control monolayers were **not** exposed to Lipiodol*
Values represent arithmetic mean (n = 10)
IOD measured in arbitrary units

Figure 2.15

Results of computerised image analysis:
Effect of Lipiodol concentrations and duration of exposure
on Lipiodol incorporation by HUVECs
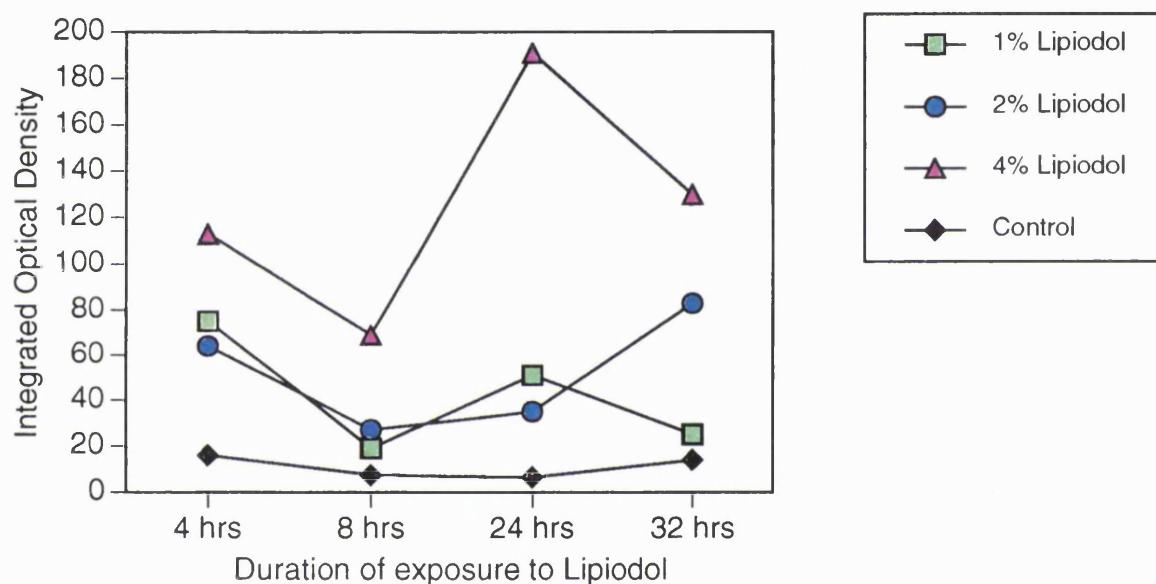
Figures represent average integrated optical density (IOD) per cell in cell monolayers
following exposure to Lipiodol (Silver nitrate stain for Lipiodol, counterstained with
*Carazzi's Haematoxylin); Control monolayers were **not** exposed to Lipiodol*
Values represent arithmetic mean (n = 10)
IOD measured in arbitrary units

2.4 CONCLUSIONS

Human liver cancer (Hep G2) cells and endothelial cells in culture incorporated Lipiodol when they were exposed to it for periods of 4 to 32 hours at concentrations similar to what may be expected in the tumour vessels. This was confirmed by light microscopy following silver nitrate impregnation. Further confirmation was provided by transmission electron microscopy of liver cancer cells, which demonstrated that the cytoplasmic lipid vesicles were membrane-bound, suggesting pinocytosis as the mechanism of incorporation.

Quantitation of Lipiodol uptake by cell monolayers (by computer-assisted analysis of optical density following silver nitrate impregnation) revealed differences between the two cell types in their patterns of Lipiodol incorporation. HepG2 demonstrated a slower initial uptake but continued to accumulate Lipiodol upto 32 hours, except at the highest concentration, where the initial uptake was more rapid, and intracellular levels remained at a plateau thereafter. Endothelial cells demonstrated a more rapid initial uptake, which was followed by a fall in intracellular levels, and then again a further rise. These differences in behaviour between the two cell types may have interesting implications if they extrapolate to the *in vivo* situation, as has been further discussed in chapter 4.

EFFECTS OF LIPIODOL INCORPORATION ON THE VIABILITY AND METABOLIC PROCESSES OF TUMOUR AND ENDOTHELIAL CELLS IN CULTURE

3.1   INTRODUCTION

Rationale for in vitro cytotoxicity studies

In vitro cytotoxicity assays have been widely used in the fields of toxicology and cancer chemotherapy. Their uses include identification of potentially toxic compounds and the mechanisms by which they exert their toxic effects, identification of potential target cell populations, determination of effective concentration ranges and relationship of concentration to exposure time (166). As Lipiodol is a vehicle for anticancer drugs and radioisotopes, the effects of Lipiodol itself on tumour cells need to be analysed before studying the effects of drug-Lipiodol complexes. The effects of water-soluble angiographic contrast

media on human cells in culture have been investigated (167) but no similar studies have been reported for Lipiodol.

The effect of a test compound on the viability of the target cell population is a fundamental parameter in toxicity testing. Cell viability is commonly measured by tests of membrane integrity, which include dye exclusion tests, enzyme release assays, and assays to measure release of a previously incorporated radioisotope. The dye Trypan Blue (and Eosin Y, Naphthalene Black and Erytrosin B to name a few others) is taken up by cells which have lost their membrane integrity and are therefore deemed non-viable. *Trypan Blue exclusion* was used a test of cell viability in this study as it is a simple, commonly used technique. Release of intracellular enzymes into the culture medium also occurs as a consequence of membrane rupture, and *Lactate dehydrogenase (LDH) release* is a useful parameter, as it is an enzyme common to a large range of cell types. In the clinical context as well, LDH levels are used to measure hepatotoxicity. LDH release was therefore used as a second test of cell viability in this study.

Of particular importance in the evaluation of an anticancer drug (or a vehicle thereof) is its effect on cell replication. Numerous techniques are available that allow estimation of cell numbers. These include colorimetric assays for measurement of the total protein content, DNA content, lysosomal activity, and enzyme activity (in particular, tetrazolium dye reduction). However, direct *counting of cell numbers -* manually or using automated counters - remains a standard technique, and has been used in this study for its simplicity.

Numerous metabolic pathways may be impaired by a substance exerting a toxic effect on a cell. In the absence of any prior data on the possible effects of a substance on cell biochemistry (as is the case with Lipiodol), it is difficult to determine which metabolic parameters should be assessed. Protein synthesis is a vital metabolic process essential for cell survival. Incorporation of amino acids into proteins (or a failure to do so) may therefore be considered as an index of cytotoxicity. In this study the *uptake of ^{3}H-leucine by cell monolayers* was measured, before and after exposure to Lipiodol, to assess the effects (if any) of Lipiodol on cellular metabolic processes.

3.2 MATERIALS AND METHODS

The technique used for culturing Hep G2 cells in tissue culture chamber slides has been outlined in Chapter 2. Cells were seeded into the wells following routine "passage", with 0.25 ml of cell suspension containing $0.5\text{-}1 \times 10^4$ cells added to each well. After overnight incubation (minimum of eight hours), the cells formed an adherent monolayer on the floor of the well i.e. on the surface of the slide. To allow for differences in the behaviour of cells related to phases of the growth cycle, two batches of cells were studied. In one batch, the medium in each well was discarded after *eight* hours of incubation and fresh medium added which contained graded amounts of Lipiodol. These cells were therefore in an early phase of growth at the time of study. In the other batch, medium containing Lipiodol was added after *forty-eight* hours of incubation, i.e. during a later phase of growth.

Of the 8 wells on each chamber-slide, 2 received medium without any Lipiodol (controls), 2 received medium with 1% Lipiodol, 2 received medium with 2% Lipiodol, and 2 received medium with 4% Lipiodol (Figure 2.3). The chamber slides were then incubated for periods of 8, 12, 24 and 32 hours, and then subjected to assessments of cell viability, cell replication and protein synthesis as outlined below.

Assessment of cell viability

Trypan Blue dye exclusion

Protocol

1   The supernatant medium was pipetted off from each well. Each cell monolayer was washed with 0.25 ml of PBS free of Ca^{++} and Mg^{++}, and the PBS was discarded.

2   0.25 ml of 10% Trypsin (0.5 ml Trypsin in 4.5 ml PBS) was added to each well, and the cells incubated at 37°C for 2-3 minutes, with intermittent inspection for cell detachment.

3   Once the cells detached from the floor of the well, the trypsin was neutralised by addition of 0.25 ml DMEM containing 20% NBCS to each well.

4   0.5 ml of the cell suspension in each well was then transferred to a small sterile container and 0.5 ml of 0.4% Trypan blue was added to it. The suspension was allowed to stand for 5 minutes.

5   Using a pipette, a small amount of the cell suspension was transferred to the counting chambers of a standard haemocytometer, for a manual cell count as outlined subsequently in this chapter.

Lactate dehydrogenase (LDH) release

At the commencement of the LDH release assay, care was taken to seed identical volumes of cell suspension (1×10^4 cells) into each well, so that given identical culture conditions, each well could be expected to have the same number of cells. After exposure to Lipiodol, the supernatent from each well was collected, and subjected to measurement of its LDH level. Standard LDH assay kits (Boehringer Mannheim) were used. These work on the basis of the following reaction:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+$$
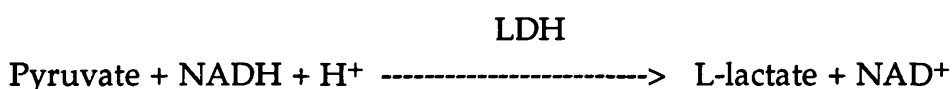
Protocol

1   Preparation of reagent: 30 reagent tablets (NADH 0.18 mmol/l) were dissolved in 90 ml of Substrate/Buffer solution (Pyruvate 0.6 mmol/l, Phosphate buffer 50 mmol/l, pH 7.5) less than 5 hours prior to use, and brought to the assay temperature (25°C).

2   The spectrophotometer was set for a wavelength of 340 nm

3   0.033 ml of culture medium (supernatent from each well) was added to 1 ml of reagent solution in a spectrophotometer cuvette. After 30 seconds, the initial absorbance was read on the spectrophotometer and the readings were repeated at exactly 1, 2 and 3 minutes.

4   The mean absorbance change per minute (ΔA_{340}/min) was determined by calculating the arithmetic mean of the three readings [The cuvette provided a 1 cm light path. The mean absorbance decrease per minute (measured against air) that the light underwent in passing through the cuvette indicated the amount of LDH in the solution].

5   The level of LDH in the sample was calculated as follows:
4921 x ΔA_{340}/min = Level of LDH in Units/litre

Cell counts: Effect on cell numbers

As outlined above in steps 1 to 5 of the protocol for the Trypan Blue dye exclusion test, cell monolayers were exposed to trypsin to create a cell suspension, and a small amount of the suspension transferred to the two counting chambers of a standard haemocytometer. Each 1 mm x 1 mm square of the haemocytometer, with cover slip in place, represents a volume of 0.1 mm^3. For each sample, 10 squares were counted. The cell concentration per ml was calculated using the formula:

Cells per ml = Average count per square x 10^4

As the total volume of cell suspension obtained from each well was 1 ml, the cell count per ml represented the total cell number in that well. From the cell counts, growth curves were constructed for each set of cultures.

Assessment of protein synthesis: Tritiated leucine uptake

Cells were cultured in tissue culture chamber slides, as outlined earlier, and exposed to graded concentrations of Lipiodol while in their early . and plateau phases of growth.

Protocol

1   At the end of the period of exposure to Lipiodol, the medium in each well was discarded, and the cell monolayer washed three times

93

with PBS.

2   Tritiated leucine (sterile aqueous solution of L-[4,5-^{3}H]Leucine, containing 1 mCi/ml activity, manufactured by Amersham plc) was added to DMEM to achieve a concentration of 5 μCi/ml.

3   0.1 ml of the tritiated leucine/DMEM solution (i.e. 0.5 μCi of activity) was added to each well.

4   The cells were incubated at 37°C for 3 hours

5   The radioactive supernatant from each well was then discarded with due precautions, and the cell monolayer washed three times with PBS.

6   0.2 ml of 1M NaOH (sodium hydroxide) solution was added to each well and left overnight, to solubilise the cellular protein.

7   The following day, 0.22 ml of 1M HCl (hydrochloric acid) was added to each well to neutralise the alkali and slightly acidify the contents.

8   The content of each well was then transferred to a minivial, and 5 mls scintillant added to each minivial. The scintillant was composed of 2.5 g Diphenyloxazole in 500 ml Toluene and 250 ml Triton-X-100.

9   The vials were transferred to a Wallac Automatic Beta Counter with pre-programmed quench correction. The activity in the samples was counted for 5 minutes each, and measured in DPM (disintegrations per minute)

Statistical analysis of all data was carried out on an Apple Macintosh computer using the software program 'StatWorks Version 1.2' (© Cricket Software Inc. 1985). Testing for significance was carried out using the Mann Whitney U and Wilcoxon Signed Rank tests for non-parametric data, unless otherwise specified.

3.3 RESULTS

Effect of Lipiodol on cell viability

Trypan Blue dye exclusion

Results of the Trypan Blue dye exclusion test are listed in Tables 3.1 (A and B), and have been graphically summarised in Figure 3.1 (A and B). Lipiodol was found not to have any statistically significant effect on cell viability as measured by Trypan Blue dye exclusion.

Table 3.1 A

Effect of Lipiodol on cell viability as measured by the Trypan Blue dye exclusion test: Hep G2 cells in an early phase of growth

[Numbers represent percentage of cells viable: Mean ± Standard deviation (n = 5)]

<table>
<tr><td></td><td colspan="4">Duration of exposure to Lipiodol</td></tr>
<tr><td></td><td>4 hours</td><td>8 hours</td><td>24 hours</td><td>32 hours</td></tr>
<tr><td>Control</td><td>88.0 ± 8.9</td><td>90.4 ± 5.7</td><td>94.8 ± 2.0</td><td>98.8 ± 1.9</td></tr>
<tr><td>1% Lipiodol</td><td>83.8 ± 4.0</td><td>94.2 ± 6.2</td><td>90.6 ± 2.4</td><td>95.6 ± 2.7</td></tr>
<tr><td>2% Lipiodol</td><td>97.8 ± 3.4</td><td>96.6 ± 3.3</td><td>94.8 ± 2.9</td><td>95.6 ± 2.7</td></tr>
<tr><td>4% Lipiodol</td><td>94.8 ± 3.2</td><td>97.0 ± 2.7</td><td>90.8 ± 8.6</td><td>95.0 ± 4.6</td></tr>
</table>

Table 3.1 B

Effect of Lipiodol on cell viability as measured by the Trypan Blue dye exclusion test: Hep G2 cells in a late phase of growth

[Numbers represent percentage of cells viable: Mean ± Standard deviation (n = 6)]

<table>
<tr><td></td><td colspan="4">Duration of exposure to Lipiodol</td></tr>
<tr><td></td><td>4 hours</td><td>8 hours</td><td>24 hours</td><td>32 hours</td></tr>
<tr><td>Control</td><td>81.0 ± 13.3</td><td>90.0 ± 4.4</td><td>94.5 ± 4.2</td><td>91.0 ± 6.4</td></tr>
<tr><td>1% Lipiodol</td><td>88.8 ± 8.3</td><td>82.6 ± 12.8</td><td>92.1 ± 6.2</td><td>86.3 ± 12.0</td></tr>
<tr><td>2% Lipiodol</td><td>89.5 ± 7.7</td><td>86.2 ± 2.8</td><td>94.1 ± 3.1</td><td>93.7 ± 6.5</td></tr>
<tr><td>4% Lipiodol</td><td>87.0 ± 1.9</td><td>85.3 ± 9.2</td><td>93.2 ± 5.7</td><td>94.3 ± 5.7</td></tr>
</table>

Figure 3.1 A
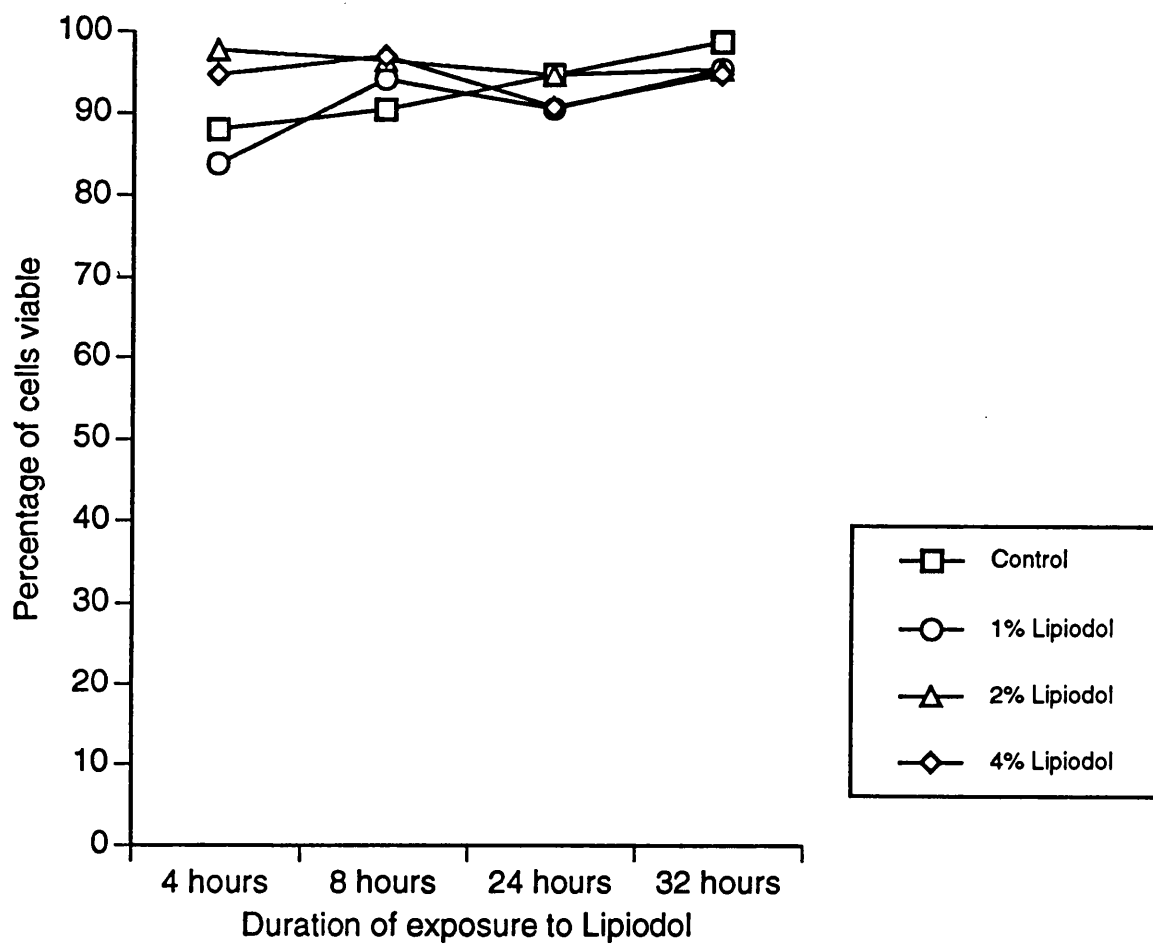
Effect of Lipiodol on cell viability
(Trypan Blue dye exclusion test)

HepG2 in an early phase of growth
Lipiodol added after 8 hours of incubation

Values indicate arithmetic mean; n = 5

Figure 3.1 B
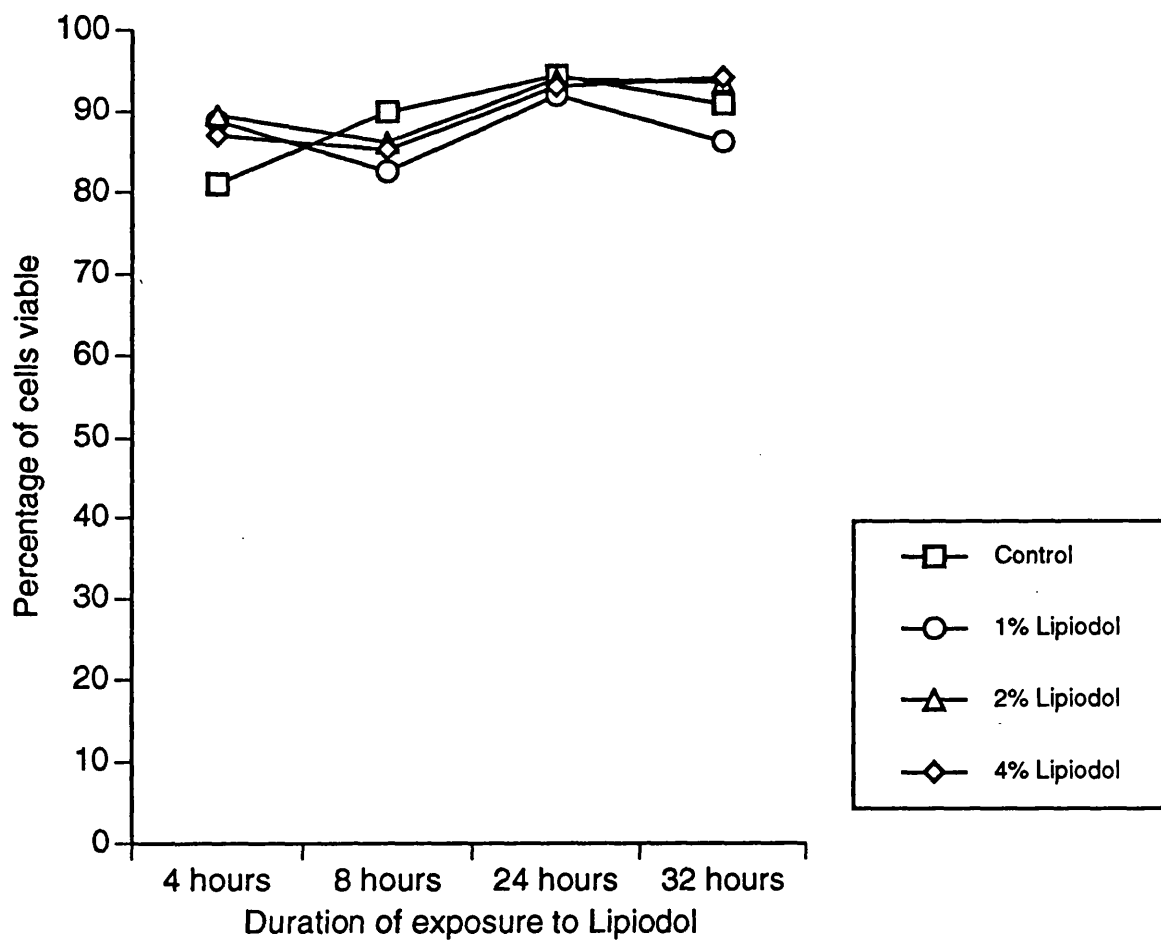
Effect of Lipiodol on cell viability
(Trypan Blue dye exclusion test)

HepG2 in a late phase of growth
Lipiodol added after 48 hours of incubation

Values indicate arithmetic mean; n = 6

LDH (Lactate dehydrogenase release)

The effect of Lipiodol on LDH release by the cell monolayers is depicted in Table 3.2 (A and B), and has been graphically summarised in Figure 3.2 (A and B). Lipiodol was found not to have any statistically significant effect on cell viability as measured by LDH release.

Table 3.2 A

Effect of Lipiodol on cell viability as measured by LDH release: Hep G2 cells in an early phase of growth

[Numbers represent level of LDH in culture medium (in Units/litre): Mean ± Standard deviation (n = 5)]

<table>
<tr><td colspan="5" align="center">Duration of exposure to Lipiodol</td></tr>
<tr><td></td><td>4 hours</td><td>8 hours</td><td>24 hours</td><td>32 hours</td></tr>
<tr><td>Control</td><td>28.4 ± 4.7</td><td>23.6 ± 2.0</td><td>33.4 ± 11.0</td><td>26.5 ± 8.5</td></tr>
<tr><td>1% Lipiodol</td><td>27.5 ± 2.4</td><td>43.3 ± 21.8</td><td>45.2 ± 2.4</td><td>31.4 ± 2.4</td></tr>
<tr><td>2% Lipiodol</td><td>23.5 ± 3.7</td><td>28.5 ± 3.7</td><td>38.3 ± 3.7</td><td>42.2 ± 6.7</td></tr>
<tr><td>4% Lipiodol</td><td>32.4 ± 6.6</td><td>52.1 ± 35.8</td><td>33.4 ± 12.9</td><td>48.2 ± 13.3</td></tr>
</table>

Table 3.2 B

Effect of Lipiodol on cell viability as measured by LDH release: Hep G2 cells in a late phase of growth

[Numbers represent level of LDH in culture medium (in Units/litre): Mean ± Standard deviation (n = 4)]

<table>
<tr><td colspan="5" align="center">Duration of exposure to Lipiodol</td></tr>
<tr><td></td><td>4 hours</td><td>8 hours</td><td>24 hours</td><td>32 hours</td></tr>
<tr><td>Control</td><td>24.6 ± 0.0</td><td>24.6 ± 0.0</td><td>40.5 ± 2.1</td><td>93.5 ± 14.3</td></tr>
<tr><td>1% Lipiodol</td><td>29.5 ± 0.0</td><td>28.3 ± 2.1</td><td>40.5 ± 4.0</td><td>62.7 ± 12.2</td></tr>
<tr><td>2% Lipiodol</td><td>33.2 ± 4.0</td><td>28.3 ± 2.1</td><td>39.3 ± 3.5</td><td>56.5 ± 2.5</td></tr>
<tr><td>4% Lipiodol</td><td>38.1 ± 2.0</td><td>45.5 ± 12.7</td><td>43.0 ± 6.4</td><td>61.5 ± 5.5</td></tr>
</table>

[N. B. The LDH level in pure culture medium (DMEM with FCS) without any cells was 9.8 U/l. Normal human serum levels are 120-240 U/l.]

Figure 3.2 A
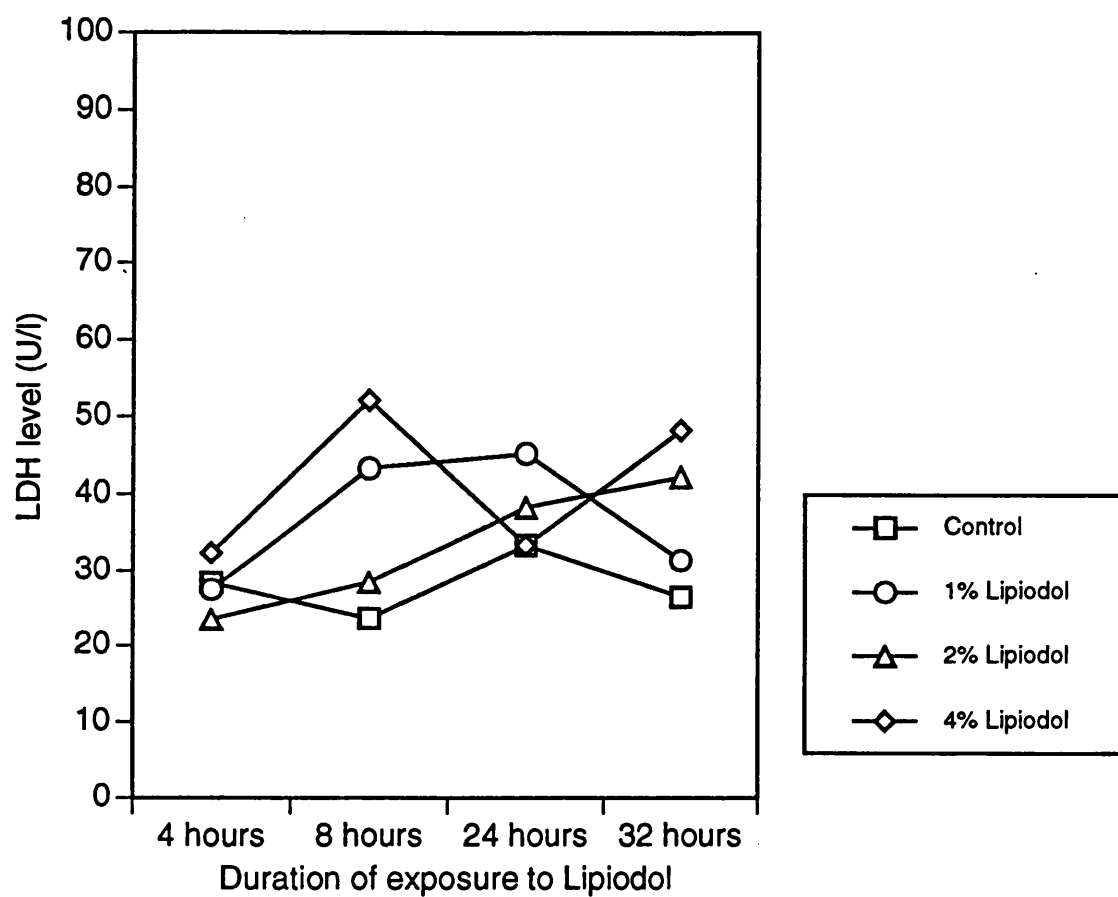
LDH release on exposure to Lipiodol

HepG2 cells in an early phase of growth

Lipiodol added at 8 hours of incubation

Values represent arithmetic mean; n = 5

Figure 3.2 B
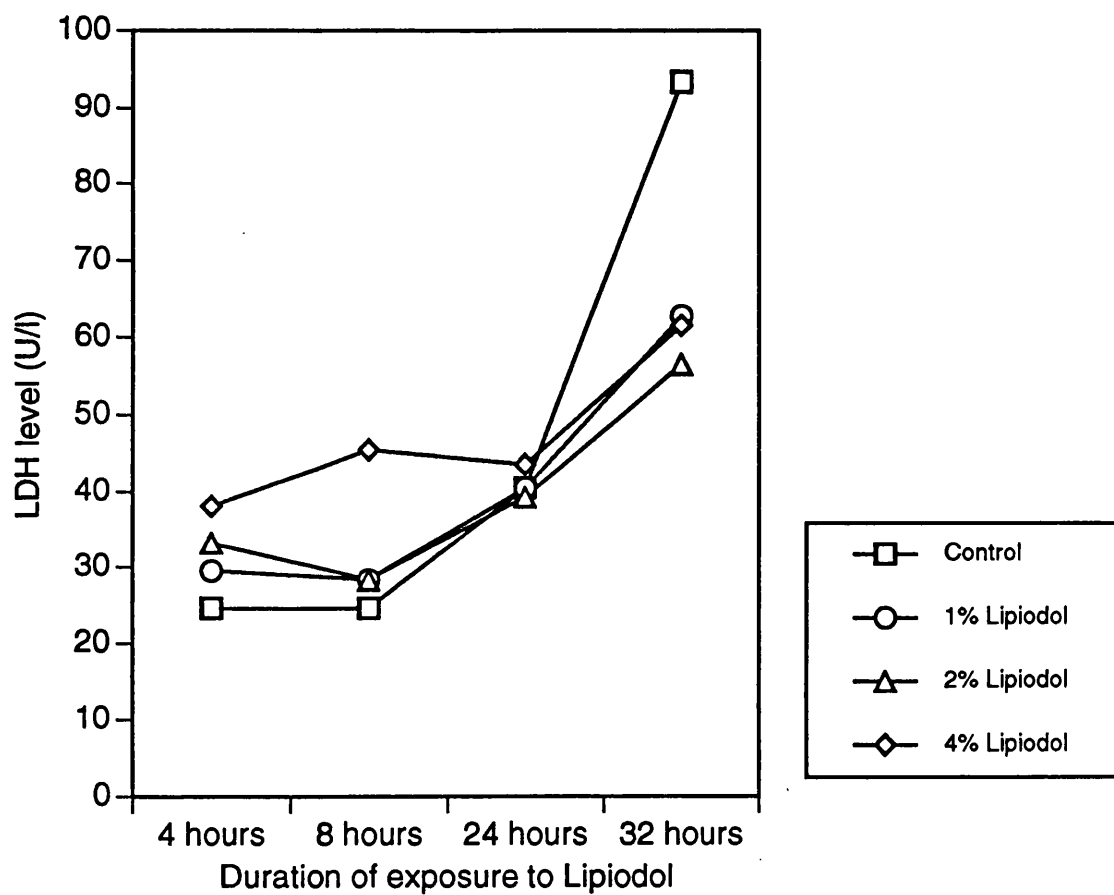
LDH release on exposure to Lipiodol
HepG2 cells in a late phase of growth

Lipiodol added at 48 hours of incubation

Values represent arithmetic mean; n = 4

Effect of Lipiodol on cell numbers

Results of the cell counts are listed in Table 3.3 (A and B) and shown graphically in Figure 3.4 (A and B). Lipiodol was found not to have any statistically significant effect on cell numbers.

Table 3.3 A

Effect of Lipiodol on cell numbers: Hep G2 in an early phase of growth

- *Values represent number of cells x 10^4*
- *n = 5*
- *Mean ± Standard deviation shown for each time period*
- *1 x 10^4 cells added to each well at commencement; Lipiodol added after 8 hours of incubation*

Duration of exposure to Lipiodol

	4 hours	8 hours	24 hours	32 hours
Control	1.75 ± 0.46	1.04 ± 0.22	2.54 ± 0.63	3.02 ± 0.45
1% Lipiodol	1.68 ± 0.32	1.42 ± 0.48	2.64 ± 0.22	2.52 ± 0.32
2% Lipiodol	1.92 ± 0.83	1.28 ± 0.49	2.18 ± 0.24	2.22 ± 0.45
4% Lipiodol	1.94 ± 0.19	1.16 ± 0.30	1.90 ± 0.21	1.18 ± 0.07

Table 3.3 B

Effect of Lipiodol on cell numbers: Hep G2 in a late phase of growth:

- *Values represent number of cells x 10^4*
- *n = 6*
- *Mean ± Standard deviation shown for each time period*
- *0.6 x 10^4 cells added to each well at commencement; Lipiodol added after 48 hours of incubation*

Duration of exposure to Lipiodol

	4 hours	8 hours	24 hours	32 hours
Control	1.75 ± 0.77	1.74 ± 0.34	1.35 ± 0.44	0.84 ± 0.18
1% Lipiodol	1.08 ± 0.25	1.17 ± 0.31	1.43 ± 0.27	1.60 ± 1.21
2% Lipiodol	1.09 ± 0.48	1.31 ± 0.35	1.45 ± 0.49	0.68 ± 0.25
4% Lipiodol	1.10 ± 0.37	1.48 ± 0.75	1.62 ± 0.76	1.00 ± 0.37

Figure 3.3 A
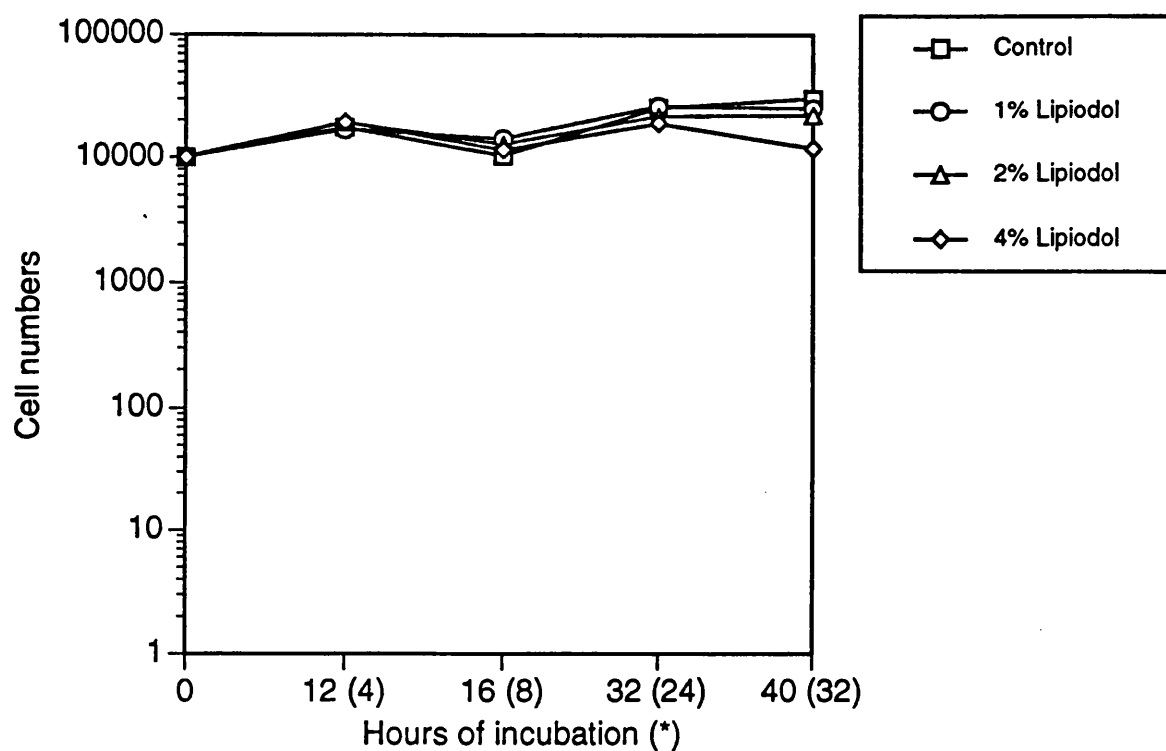
Effect of Lipiodol on cell numbers
(depicted on a logarithmic scale)
HepG2 cells in an early phase of growth

*Lipiodol added at 8 hours (Period of
exposure to Lipiodol shown in parentheses)

Figure 3.3 B
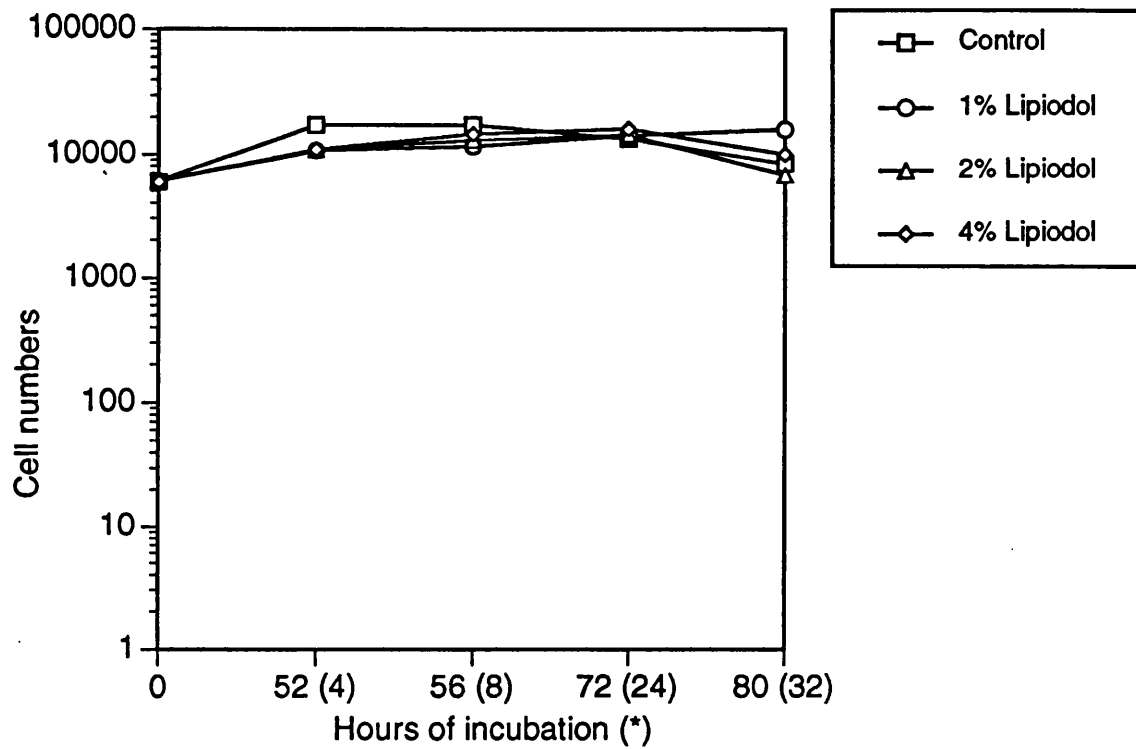
Effect of Lipiodol on cell numbers
(depicted on a logarithmic scale)
HepG2 cells in a late phase of growth

* Lipiodol added at 48 hours (Period of
exposure to Lipiodol shown in parentheses)

Effect of Lipiodol on tritiated Leucine uptake

The effect of exposure to Lipiodol on the uptake of tritiated Leucine by Hep G2 cells is shown in Tables 3.4 (A and B), and Figure 3.4 (A and B). Lipiodol was found not to have any statistically significant effect on the cellular uptake of tritiated Leucine.

Table 3.4 A

Effect of Lipiodol on ^{3}H-labelled Leucine uptake: HepG2 in an early phase of growth

- *Values represent activity in cell monolayers, counted in DPM (disintegrations per minute)*
- *n = 4*
- *Mean ± Standard deviation shown for each time period*
- *0.6 x 10^4 cells added to each well at commencement*

<table>
<tr><td></td><td colspan="4">Duration of exposure to Lipiodol</td></tr>
<tr><td></td><td>4 hours</td><td>8 hours</td><td>24 hours</td><td>32 hours</td></tr>
<tr><td>Control</td><td>1036 ± 345</td><td>2137 ± 545</td><td>4237 ± 562</td><td>2561 ± 250</td></tr>
<tr><td>1% Lipiodol</td><td>788 ± 191</td><td>1870 ± 623</td><td>4986 ± 960</td><td>3170 ± 708</td></tr>
<tr><td>2% Lipiodol</td><td>960 ± 121</td><td>1936 ± 284</td><td>5455 ± 996</td><td>3944 ± 380</td></tr>
<tr><td>4% Lipiodol</td><td>1054 ± 79</td><td>1845 ± 301</td><td>4310 ± 1204</td><td>3041 ± 334</td></tr>
</table>

Table 3.4 B

Effect of Lipiodol on ^{3}H-labelled Leucine uptake: HepG2 in a later phase of growth

- *Values represent activity in cell monolayers, counted in DPM (disintegrations per minute)*
- *n = 4*
- *Mean ± Standard deviation shown for each time period*
- *0.6 x 10^4 cells added to each well at commencement*

<table>
<tr><td></td><td colspan="4">Duration of exposure to Lipiodol</td></tr>
<tr><td></td><td>4 hours</td><td>8 hours</td><td>24 hours</td><td>32 hours</td></tr>
<tr><td>Control</td><td>7743 ± 2962</td><td>5449 ± 1435</td><td>4348 ± 849</td><td>2553 ± 193</td></tr>
<tr><td>1% Lipiodol</td><td>6256 ± 2102</td><td>5462 ± 368</td><td>5349 ± 1046</td><td>3157 ± 607</td></tr>
<tr><td>2% Lipiodol</td><td>5884 ± 779</td><td>6060 ± 303</td><td>5201 ± 1203</td><td>3545 ± 490</td></tr>
<tr><td>4% Lipiodol</td><td>7894 ± 1182</td><td>7970 ± 1666</td><td>7715 ± 1167</td><td>4133 ± 593</td></tr>
</table>

Figure 3.4 A
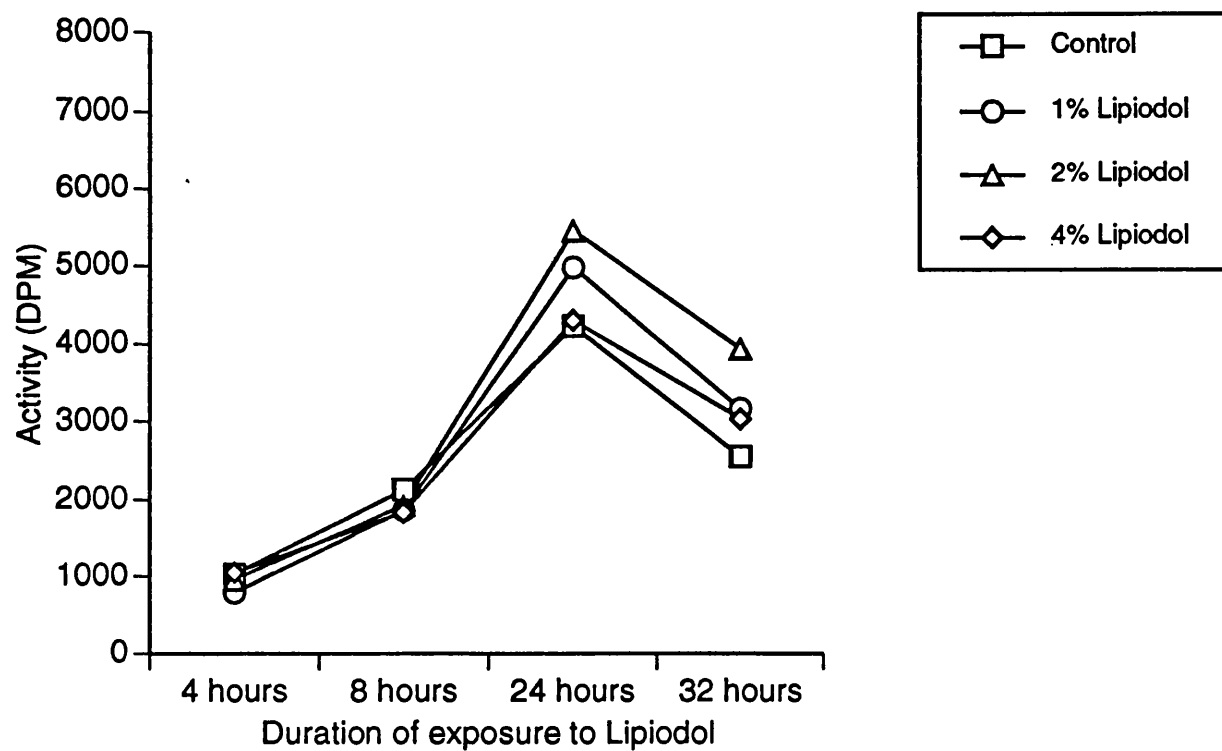
Effect of Lipiodol on ^{3}H-labelled Leucine uptake

HepG2 in the early phase of growth
Lipiodol added at 8 hours of incubation
Values represent arithmetic mean; n = 4

Figure 3.4 B
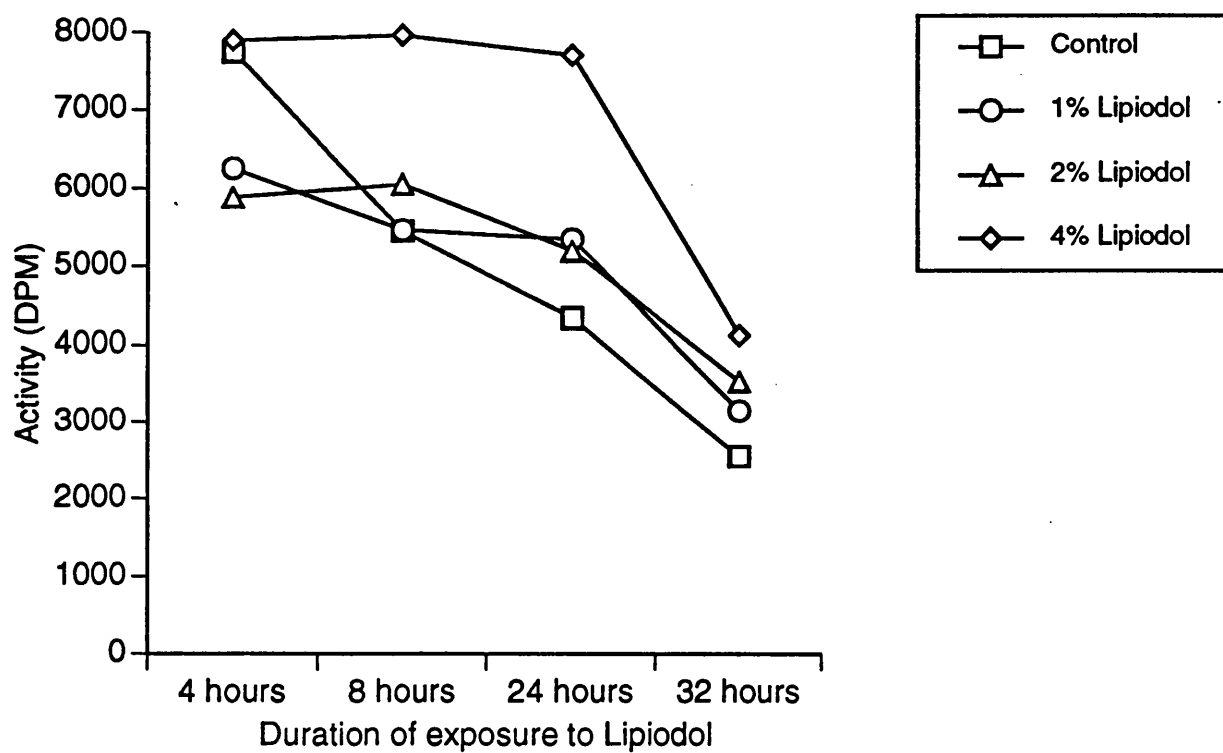
Effect of Lipiodol on ^{3}H-labelled Leucine uptake

HepG2 in the late phase of growth
Lipiodol added at 48 hours of incubation
Values represent arithmetic mean; n = 4

3.4 CONCLUSIONS

Cultures of the liver tumour cell line HepG2 were exposed to Lipiodol for periods of 4-32 hours, at concentrations akin to what may be expected in the hepatic sinusoids. The cells suffered no significant adverse effects in terms of cell viability, cell replication and amino acid (^{3}H-labelled Leucine) uptake. This would suggest that Lipiodol itself has no cytotoxic effects at these concentrations, and to achieve a tumoricidal effect it would have to be conjugated to a cytotoxic drug or radio-isotope.

ANATOMICAL LOCALISATION OF LIPIODOL IN TUMOURS:
HISTOLOGIC STUDY OF HEPATOCELLULAR CARCINOMAS
PERFUSED WITH LIPIODOL IN VIVO OR EX VIVO

4.1 INTRODUCTION

4.2 MATERIALS AND METHODS

Histologic assessment of resected tumours

Study of HCCs in explant livers: Ex vivo Lipiodol perfusion

Study of HCCs in explant livers: Lipiodol angiography prior to
transplantation

4.3 RESULTS

Silver nitrate impregnation is a selective staining technique for Lipiodol

Anatomic localisation of Lipiodol in resected tumours

Retention of Lipiodol in incidental HCCs in explant livers

Retention of Lipiodol in a tumour perfused ex vivo

4.4 CONCLUSIONS

Why is Lipiodol selectively retained by HCCs?

The significance of Lipiodol incorporation by tumour and endothelial cells

The potential role of Lipiodol CT prior to liver transplantation

---

4.1 INTRODUCTION

Lipiodol administration (at diagnostic angiography, or for adjuvant
therapy, combined with a cytotoxic drug or radioisotope) is usually
carried out several days to weeks prior to surgical resection. Thus,
histology of the resected tumour captures the anatomic picture at a point
in time many hours from the critical moment of Lipiodol injection.
Histopathologic assessment of tumours resected following arterial
administration of Lipiodol was carried out by Arnold et al in 1992, in an
attempt to study Lipiodol localisation in HCCs (149). The conjugation of
cytotoxic agents makes it difficult to assess the effect of Lipiodol *alone* on

the tumour tissue.

Recognising these drawbacks, Lipiodol retention in human HCCs was studied in three different settings:

(1) Resected HCCs (targeted with adjuvant Lipiodol-epirubicin prior to surgery) were subjected to histologic analysis following silver nitrate impregnation.

(2) Prospective OLT recipients were administered hepatic arterial Lipiodol at the time of routine angiography. After transplantation, the explant livers were X-rayed, and biopsies taken from areas of focal Lipiodol retention. This allowed assessment of Lipiodol retention in the absence of conjugated cytotoxic agents.

(3) Explant livers removed freshly from recipients of orthotopic liver transplantation (OLT) were flushed with Lipiodol via the hepatic artery within minutes of removal in the expectation that any foci of HCC present in these livers would take up Lipiodol [None of these patients was known to have a HCC, but cirrhosis irrespective of its aetiology predisposes to HCC (182), and a significant proportion of OLT recipients harbour foci of 'incidental' HCC in their livers at the time of transplantation (41)]. Tissue was taken from all areas of focal Lipiodol uptake (as judged from soft tissue X-ray pictures of the liver specimens) in the expectation that histologic analysis of such tumour foci may yield an insight into the events that occur immediately after Lipiodol injection.

4.2   MATERIALS AND METHODS

Histologic assessment of resected tumours

Over a period of eighteen months at the Royal Free Hospital, eight patients undergoing liver resection for primary HCC were administered adjuvant hepatic arterial Lipiodol-epirubicin prior to surgical resection of their tumours. All patients received 10 ml Lipiodol emulsified with the required dose of epirubicin (with Urografin) by agitation in a Pulsatronic ultrasonic agitator for 5 minutes, less than 6 hours prior to injection. The particulars of these patients are summarised in Table 4.1.

Table 4.1

Patients undergoing resection of HCCs following intra-arterial Lipiodol-epirubicin therapy

Age/Sex	Size, location and differentiation of HCC	Lipiodol-Epirubicin dosage & timing	Surgical procedure
56 yrs Male (F. E-A.)	6 cm diameter Segments VII, VIII Poorly differentiated	120 mg Epirubicin 17 days before surgery	Resection of tumour with 2 cm margin
63 yrs Male (G. D.)	5 cm diameter Segment IV Fully necrosed	80 mg Epirubicin 50 days before surgery	Resection of tumour with 1 cm margin
68 yrs Male (A. L.)	3.5 cm diameter Segment IV Well differentiated	90 mg Epirubicin 20 days before surgery	Resection of tumour with 1 cm margin
59 yrs Male (R. K.)	6 cm diameter Right lobe Well differentiated	Only Lipiodol (no Epirubicin) 32 days before surgery	Right hemi-hepatectomy
74 yrs Male (D. D.)	11 cm diameter Right lobe Moderately differentiated	80 mg Epirubicin 21 days before surgery	Right hemi-hepatectomy
62 yrs Male (R. C.)	3 cm diameter Segment VIII Well differentiated	60 mg Epirubicin 1 day before surgery	Resection of tumour with 2 cm margin
58 yrs Male (G. G.)	4 cm diameter Segments V and VIII	80 mg Epirubicin 18 days before surgery	Resection of tumour with 2 cm margin
65 yrs Male (S. B.)	12 cm diameter Right lobe Well differentiated	70 mg Epirubicin 24 days before surgery	Right hemi-hepatectomy

In each instance, soon after the tumour was resected, several blocks of tumour tissue were removed from the specimen, and processed for histologic analysis using a technique adapted from Arnold et al (179).

Protocol:

1   The tissues were fixed by immersion in formalin (10% formaldehyde) solution for 24 hours.

2   To remove excess formalin, the blocks of tissue were soaked in a large quantity of distilled water overnight, and the water changed again in the morning.

3   The blocks were immersed in a 2.5% solution of silver nitrate for 45 minutes at room temperature in the dark.

4   The blocks were rinsed with distilled water to remove excess silver nitrate from the surface.

5   The tissues were then processed to paraffin wax using the routine process but avoiding contamination with formalin in the processing fluids.

6   Paraffin sections were cut at 3 μm and stained with haematoxylin and eosin.

Lipiodol in the tissues was stained golden brown to black by this process; nuclei were stained blue and cytoplasm red. Interpretation of all histology slides was carried out with the help of a senior Consultant Histopathologist.

Study of HCCs in explant livers: Ex vivo Lipiodol perfusion

Eleven cirrhotic explant livers removed from recipients of orthotopic liver transplantation (OLT) were flushed with Lipiodol via the hepatic artery immediately after removal. The right lobe of a healthy donor liver (the left lobe was used for a paediatric recipient) was also similarly perfused 24 hours after removal, while the liver was still viable. None of these 12 patients was suspected to have a HCC at the time, and none had received hepatic arterial Lipiodol at any time in the past. Their particulars are listed in Table 4.2

Table 4.2

Explant livers perfused with Lipiodol ex vivo

Age/Sex of patient	Underlying liver disease	Suspicion of HCC on pre-operative CT scan or angiogram	Serum AFP level (Normal: 0 - 10 kU/L
1) 41/Male	HBV & HDV cirrhosis	No	Normal
2) 9/Male	Wilson's disease	No	Normal
3) 37/Male	Healthy (donor) liver	Not investigated	Not done
4) 27/Female	Budd Chiari syndrome	No	Normal
5) 41/Male	HCV cirrhosis	No	Normal
6) 49/Female	Primary biliary cirrhosis	No	Normal
7) 36/Male	Primary sclerosing cholangitis	No	Normal
8) 42/Male	HCV cirrhosis	No	23 kU/L
9) 44/Male	Chronic active hepatitis (lupoid)	No	Normal
10) 46/Male	HCV cirrhosis	No	Normal
11) 46/Male	Primary biliary cirrhosis	No	Normal
12) 45/Female	Primary biliary cirrhosis	No	Normal

Each of the explant livers was collected in the operating theatre immediately after removal from the recipient.

Protocol:

1   The hepatic artery was cannulated, and the liver perfused with 200 ml Haemaccel over 2-3 minutes to flush out possible clots (Haemaccel is a colloidal solution for intravenous infusion, clinically used as a plasma expander).

2   Ten ml of Lipiodol emulsified by agitation in an equal volume of Haemaccel was injected over a minute into the hepatic artery

3   The artery was again flushed with 300 ml Haemaccel.

4   The liver was immersed in formalin (10% formaldehyde solution), and tranported to the Histopathology Department.

5   Following fixation overnight in formalin, the liver was cut into 1 cm thick slices in its longitudinal axis, and an X-ray was taken of each slice. The intensity and duration of exposure were modified as appropriate for soft tissue specimens (usually 20 kilovolts X 3 minutes).

6   The X-ray pictures were examined for nodules showing focal Lipiodol uptake, and a tissue sample was taken from all such areas. showing a 'suspicious' blush on X-ray.

7   All tissue samples were impregnated with silver nitrate and processed for histologic analysis (as outlined in the protocol in the preceding section, starting from step 3).

Study of HCCs in explant livers: Lipiodol angiography prior to transplantation

From July 1993 to October 1994, 37 patients with chronic liver disease underwent routine hepatic arteriography as part of elective investigation prior to their first orthotopic liver transplantation (Appendix 3). At the conclusion of arteriography, with the catheter still in situ, 10 ml Lipiodol [emulsified less than 6 hours prior to injection in an equal volume of Urografin 290 (sodium diatrizoate and meglumine diatrizoate; Schering AG) by agitation in a Pulsatron ultrasonic agitator for 5 minutes, with aseptic precautions] was injected into the common hepatic artery. When recipient hepatectomies were subsequently performed at the time of orthotopic liver transplantation 1-6 weeks later, the explant livers were cut into 1 cm thick slices, and tissue was taken for histologic analysis from all 'suspicious' nodules (i.e. > 1 cm in diameter, or with an unusually pale or dark green colour, or with a hard

tissue consistency). If a nodule was found to be a HCC, all slices of that liver were subjected to soft-tissue X-rays, and tissue was taken for histologic analysis from all foci of Lipiodol retention (refer steps 4-7 of protocol involving explant livers in the preceding section). In addition to routine histology and silver impregnation, some of the samples were subjected to transmission electron microscopy.

4.3 RESULTS

Silver nitrate impregnation is a selective staining technique for Lipiodol

The specificity of the silver nitrate stain for Lipiodol has already been mentioned in the context of *in vitro* experiments in chapter 2. This was further confirmed at the light microscopic and ultrastructural levels during the histologic study of tumour sections. When tissues that had received Lipiodol were exposed to silver nitrate, the oil was consistently stained a golden brown colour. Conversely, impregnation with silver nitrate did not stain tissues that had not received Lipiodol. Also, unlike other lipid stains, silver nitrate did not stain endogenous fat (Figure 2.5 A and B). This is probably because silver stains Lipiodol by reacting with its iodine moiety to form silver iodide (178). The localisation of Lipiodol in tumours as demonstrated by silver impregnation correlated well with the radiologic localisation on CT scans and soft tissue X rays.

Anatomic localisation of Lipiodol in resected tumours

The 8 patients undergoing liver resection all had CT scans performed 7-10 days after Lipiodol injection. These demonstrated homogeneous uptake of contrast in the smaller tumours less than 5 cm diameter, while larger lesions showed a patchy uptake. Figures 4.1 and 4.2 represent the macroscopic appearance of a large tumour compared to its soft tissue X-ray taken following resection. The patient had received hepatic arterial Lipiodol-Epirubicin prior to surgery.

Histologically, of the 8 resected tumours, there was complete necrosis of tumour tissue in 1 specimen. Each of the other 7 specimens showed
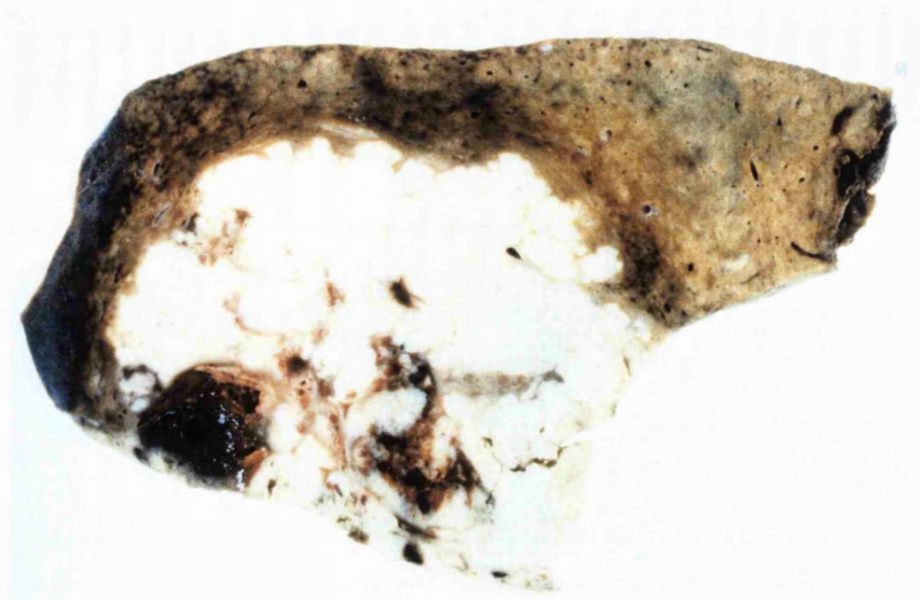
Figure 4.1: Macroscopic appearance of a large resected HCC
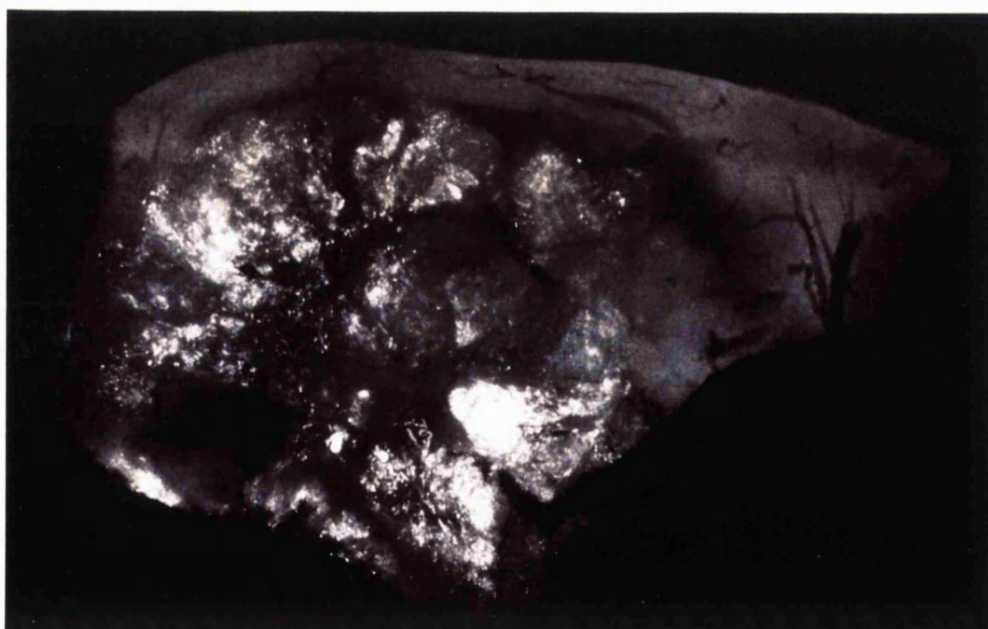
Figure 4.2: Soft-tissue X ray (20 kv x 3 mins.) of the specimen depicted in Figure 4.1
demonstrates uptake of Lipiodol by the tumour.

Lipiodol-epirubicin was administered via the hepatic artery 21 days prior to surgery
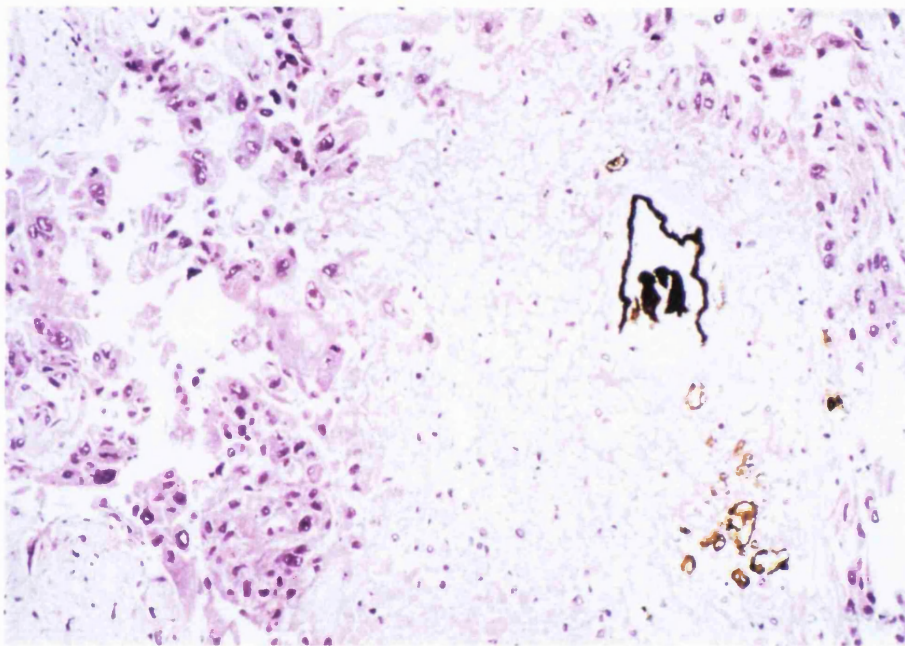
Figure 4.3: Histologic appearance of resected HCC following silver impregnation
(Lipiodol-epirubicin arterially administered prior to surgery)
demonstrates localisation of Lipiodol in tumour vessels
(Haematoxylin & eosin; magnification x 150)
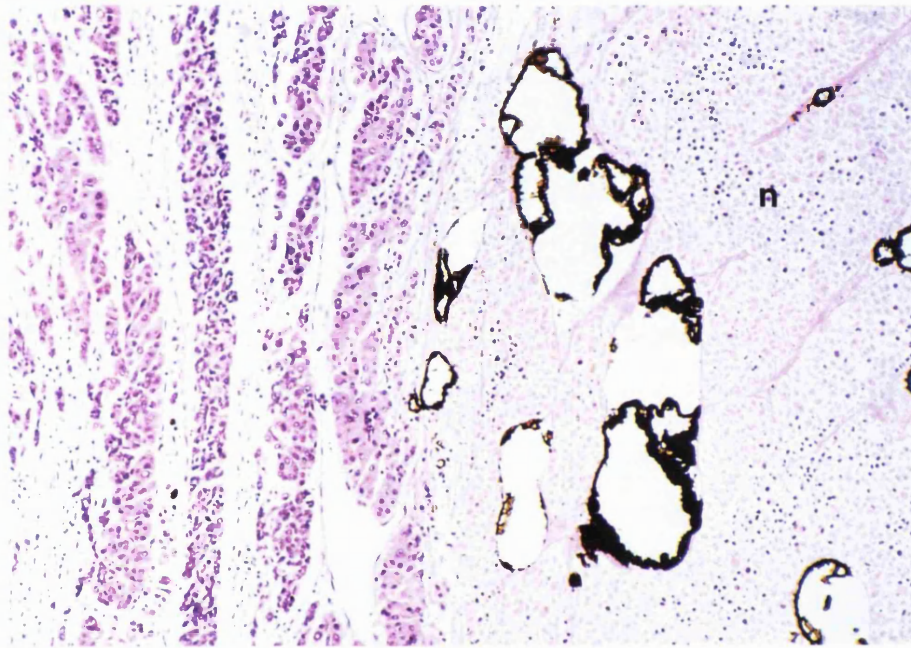
Figure 4.4: Histologic appearance of resected HCC following silver impregnation
(Lipiodol-epirubicin arterially administered prior to surgery)
demonstrates localisation of Lipiodol in tumour vessels
with areas of perivascular necrosis (n) (Haematoxylin & eosin; magnification x 100)
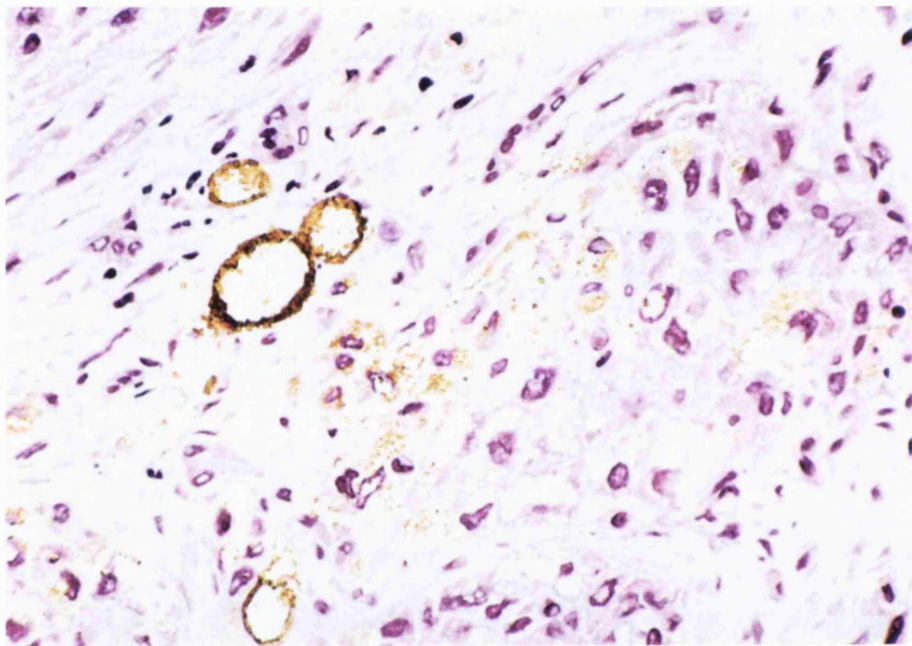
Figure 4.5: Histologic appearance of resected HCC following silver impregnation
(Lipiodol-epirubicin arterially administered prior to surgery)
demonstrates localisation of Lipiodol in tumour vessels,
and presence of intracellular Lipiodol vesicles in tumour cells
(Haematoxylin & eosin; magnification x 350)
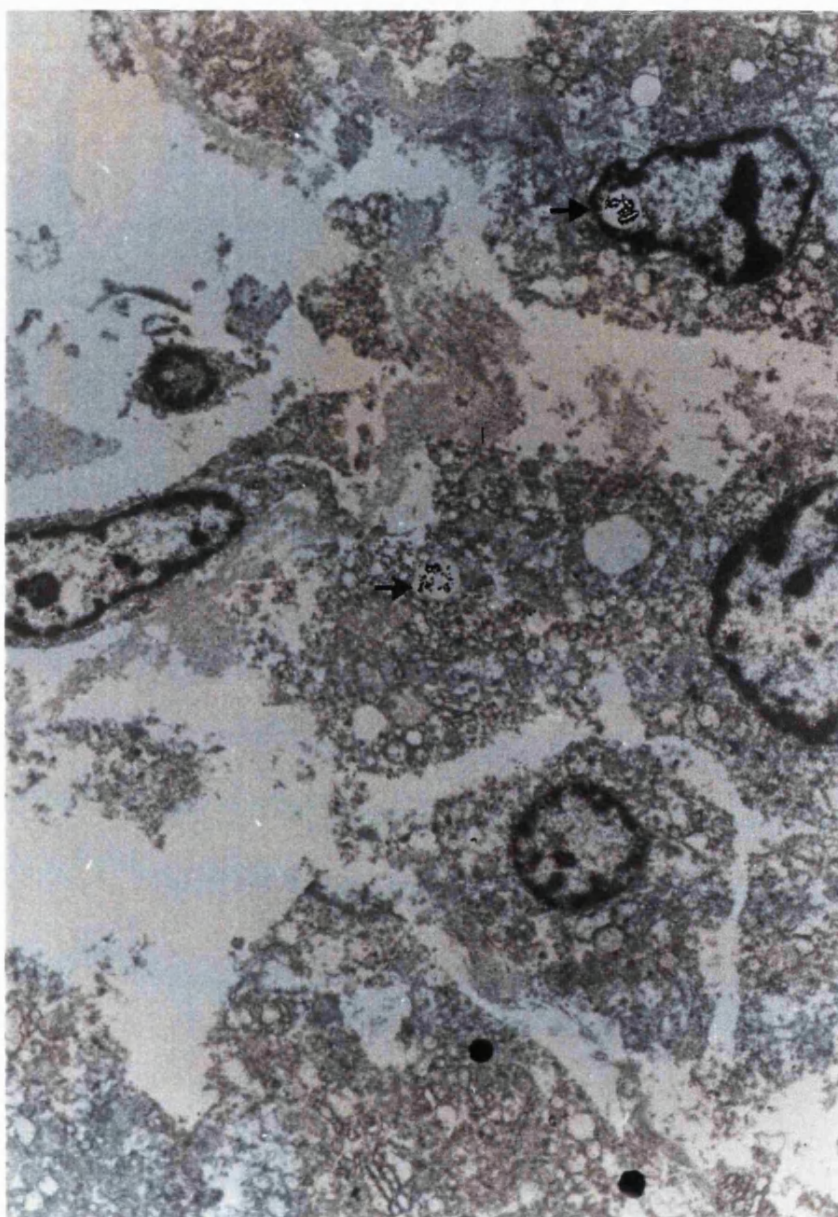
Figure 4.6: Transmission electron micrograph of resected HCC following silver impregnation. Lipiodol-epirubicin was administered prior to resection. Intracellular Lipiodol visualised as dark areas of silver impregnation within cytoplasmic vesicles, and also within nuclei (magnification x 5500)
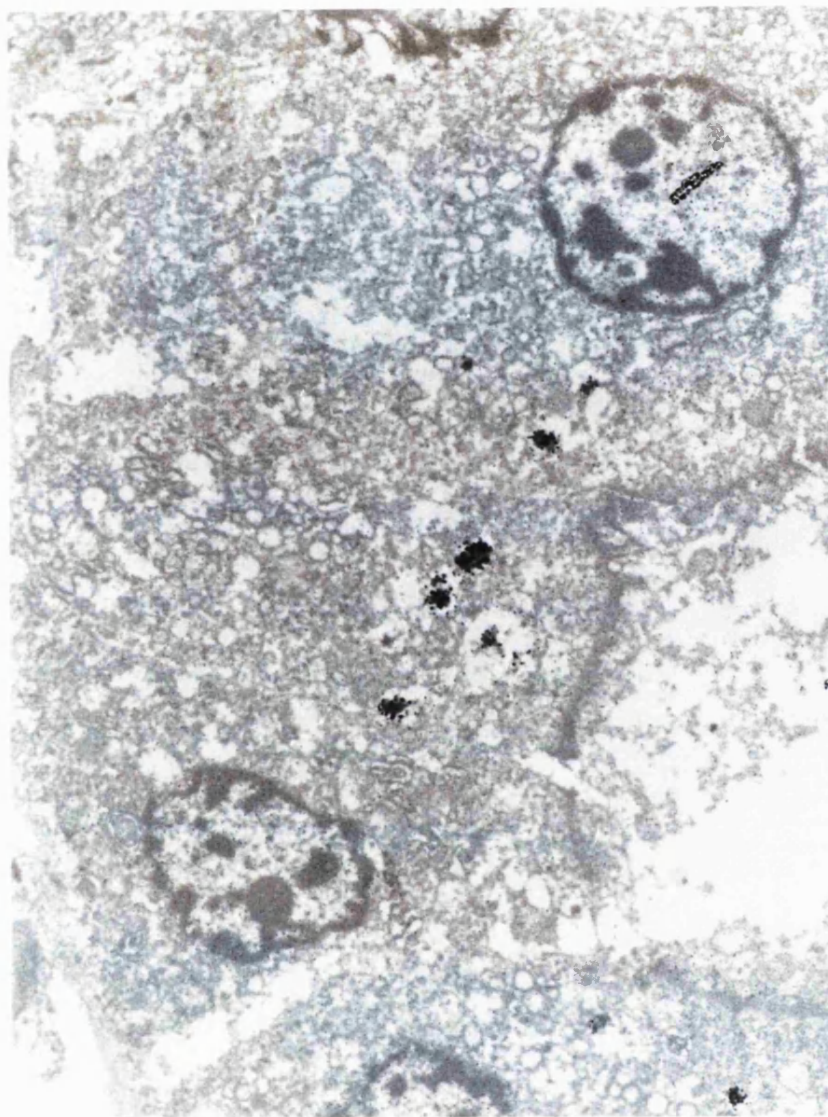
Figure 4.7: Transmission electron micrograph of resected HCC following silver impregnation. Lipiodol-epirubicin was administered prior to resection. Intracellular Lipiodol visualised as dark areas of silver impregnation within cytoplasmic vesicles, and also within nuclei (magnification x 5500)
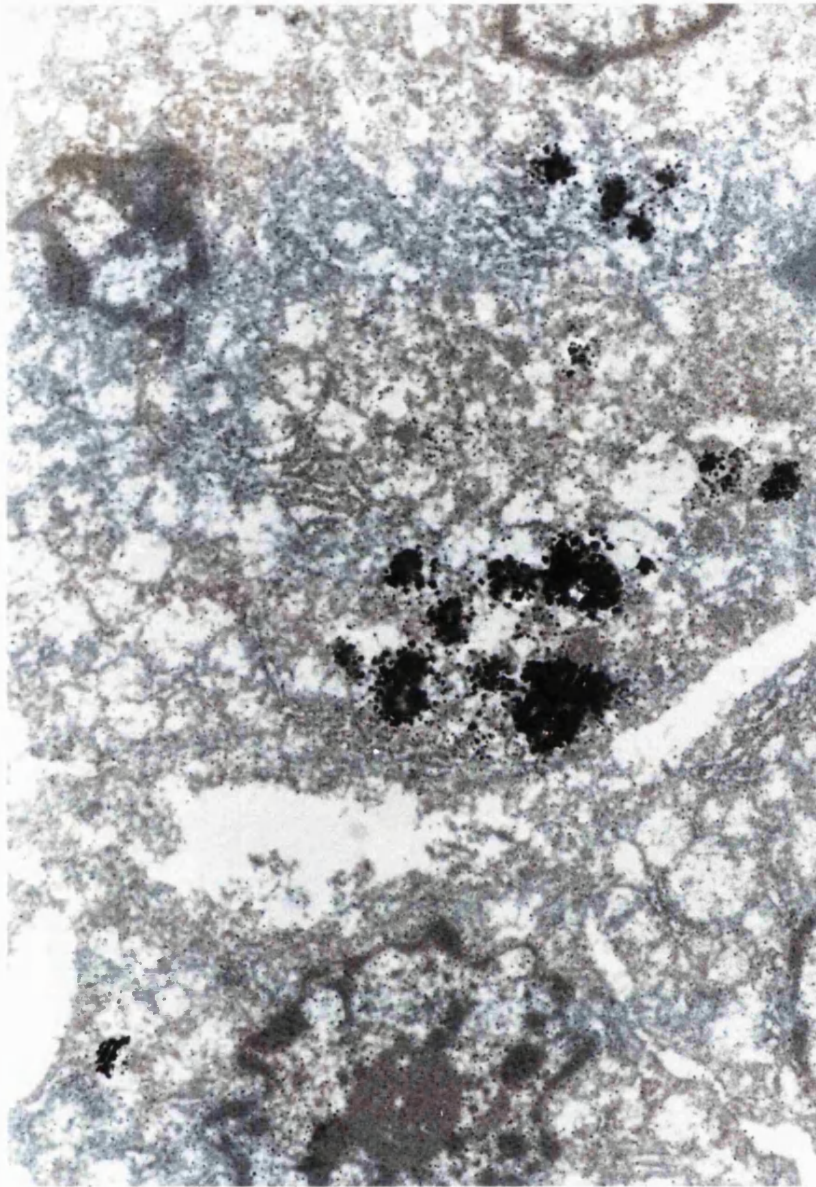
Figure 4.8: Transmission electron micrograph of resected HCC following silver impregnation. Lipiodol-epirubicin was administered prior to resection. Intracellular foci of Lipiodol visualised as dark areas of silver impregnation in the cytoplasm (magnification x 8500)

droplets of Lipiodol localised within a majority of tumour vessels (Figure 4.3). Apart from being present within the lumen of blood vessels, Lipiodol had been incorporated by the endothelial cells lining these vessels. Areas of necrosis were seen circumferentially extending around these vessels, probably reflecting the tumoricidal effect of the epirubicin conjugated to the Lipiodol. Intracellular vesicles of Lipiodol were present in scattered tumour cells lying in these necrotic regions. In some fields, coalesced pools of Lipiodol were seen to be present outwith the vascular spaces, in the necrotic areas (Figure 4.4). Some tumour cells lying away from these perivascular necrotic areas also demonstrated intracellular Lipiodol (Figure 4.5). Transmission electron microscopy of these tissue sections following silver impregnation further confirmed the presence of intracellular Lipiodol vesicles (Figures 4.6-4.8). No comparable changes or evidence of Lipiodol retention were present in the 'non-tumour' liver parenchyma. There appeared to be no visually obvious correlation between Lipiodol retention by a tumour and its degree of differentiation or the nature of the underlying liver disease.

Retention of Lipiodol in incidental HCCs in explant livers

A total of 16 foci of Lipiodol retention were discovered in 4 of the 37 livers thus examined - 15 proved to be HCCs on histologic analysis. In these lesions, Lipiodol was present in the lumen of the majority of tumour vessels. Brown intracellular vesicles of Lipiodol were seen to be present in numerous tumour cells. Intracellular Lipiodol was also present in the endothelial cells lining the tumour vessels. However, unlike the resected tumours, there were no areas of focal necrosis. In the rest of the liver, while Lipiodol droplets were occasionally present in the lumina of the larger arteries, no intracellular Lipiodol could be visualised by silver staining in any of the cell populations, despite identifiable fatty change in places.

Retention of Lipiodol in a tumour perfused ex vivo

X-rays of the 12 explant livers showed a 3 cm nodule with significant Lipiodol uptake in one patient (Figures 4.9,4.10). Histology demonstrated this to be a hitherto undetected primary HCC. Lipiodol was seen to be present in the lumen of the majority of tumour vessels. Brown

intracellular vesicles of Lipiodol were seen to be present in numerous tumour cells, which demonstrated a ballooned, foamy appearance (Figure 4.11). Intracellular Lipiodol was also present in the endothelial cells lining the tumour vessels (Figure 4.12). Transmission electron microscopy of sections from this tumour further confirmed the localisation of Lipiodol in tumour and endothelial cells (Figures 4.13-4.15). Examination of the non-tumour liver parenchyma, revealed localisation of Lipiodol in a few phagocytic cells, but no intracellular Lipiodol could be visualised in the hepatocytes or the sinusoidal endothelium by silver staining (Figure 4.16).

4.4 CONCLUSIONS

Silver nitrate impregnation of routinely fixed tissue is a simple and reliable method for identifying Lipiodol. Histologic findings on the resected tumours indicate that Lipiodol administered via the hepatic artery (1) localises *within the lumina of tumour vessels*, (2) is *incorporated by the endothelial cells lining these vessels*, and (3) is *incorporated by tumour cells*. Areas of necrosis around the vessels are probably due to the effect of the Epirubicin, though ischaemic damage from obstruction of the feeding vessels (either by embolisation of Lipiodol droplets, or due to Lipiodol-mediated endothelial injury) may also play a role.

The incidental HCCs detected in the explant livers perfused with Lipiodol (*without* epirubicin) at angiography showed little evidence of tumour necrosis - which indicates that Lipiodol *per se* has no tumoricidal effects. But they uniformly demonstrated Lipiodol droplets *within the lumina of tumour vessels, within endothelial cells lining the vesels, and within tumour cells*. However, these tumours were resected 1-6 weeks *after* Lipiodol administration.

The findings from the tumour perfused *ex vivo* are therefore of particular significance, as they probably reflect events that occur *in vivo* imediately after Lipiodol reaches the tumour vessels. This tumour, at the time of Lipiodol perfusion, had undoubtedly suffered a period of warm ischaemia, but the tumour cells were likely to be still viable. Thus, the presence of *intracellular* Lipiodol vesicles within the tumour

Figure 4.9: Macroscopic appaearance of an explant liver, which was found to contain a 3 cm nodule, which retained Lipiodol on ex-vivo perfusion

Figure 4.10: Soft-tissue X ray of explant liver shown in Figure 4.9, which demonstrates Lipiodol retention by the 3 cm nodule. Histology confirmed this as a hitherto undetected HCC.
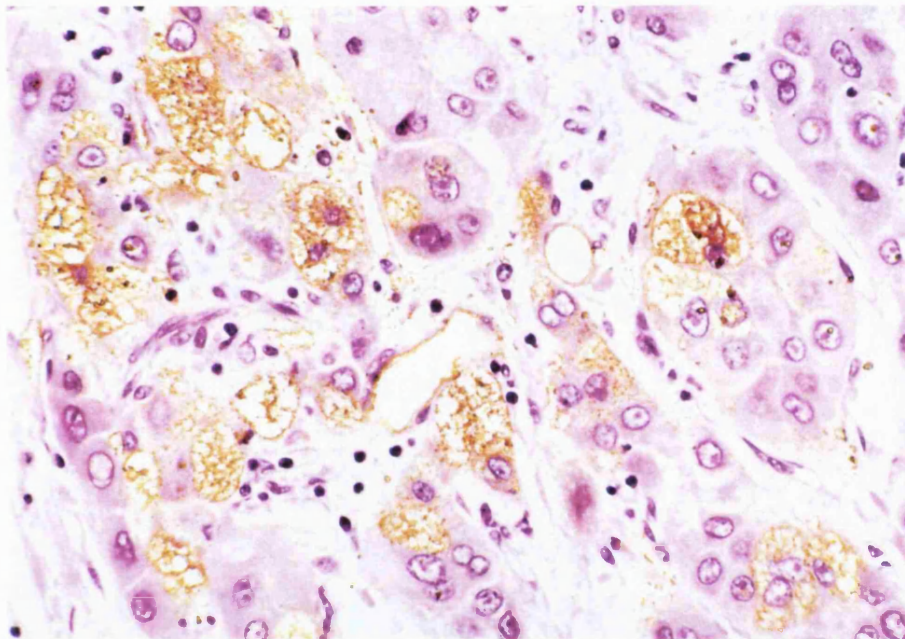
Figure 4.11: Histologic appearance of incidental HCC in explant liver,
where Lipiodol was administered ex vivo within 30 minutes of removal.
Lipiodol vesicles present within tumour cells, some of which show ballooning
(Silver impregnation followed by haematoxylin & eosin; magnification x 350)
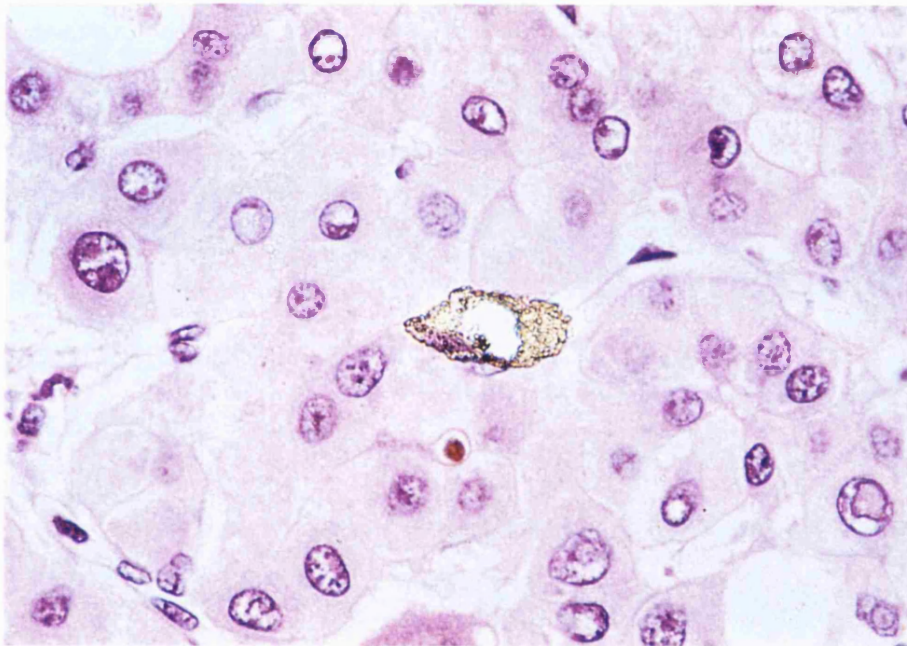
Figure 4.12: Histologic appearance of incidental HCC in explant liver,
where Lipiodol was administered ex vivo within 30 minutes of removal.
Lipiodol present within endothelial cells lining a tumour vessel
(Silver impregnation followed by haematoxylin & eosin; magnification x 550)
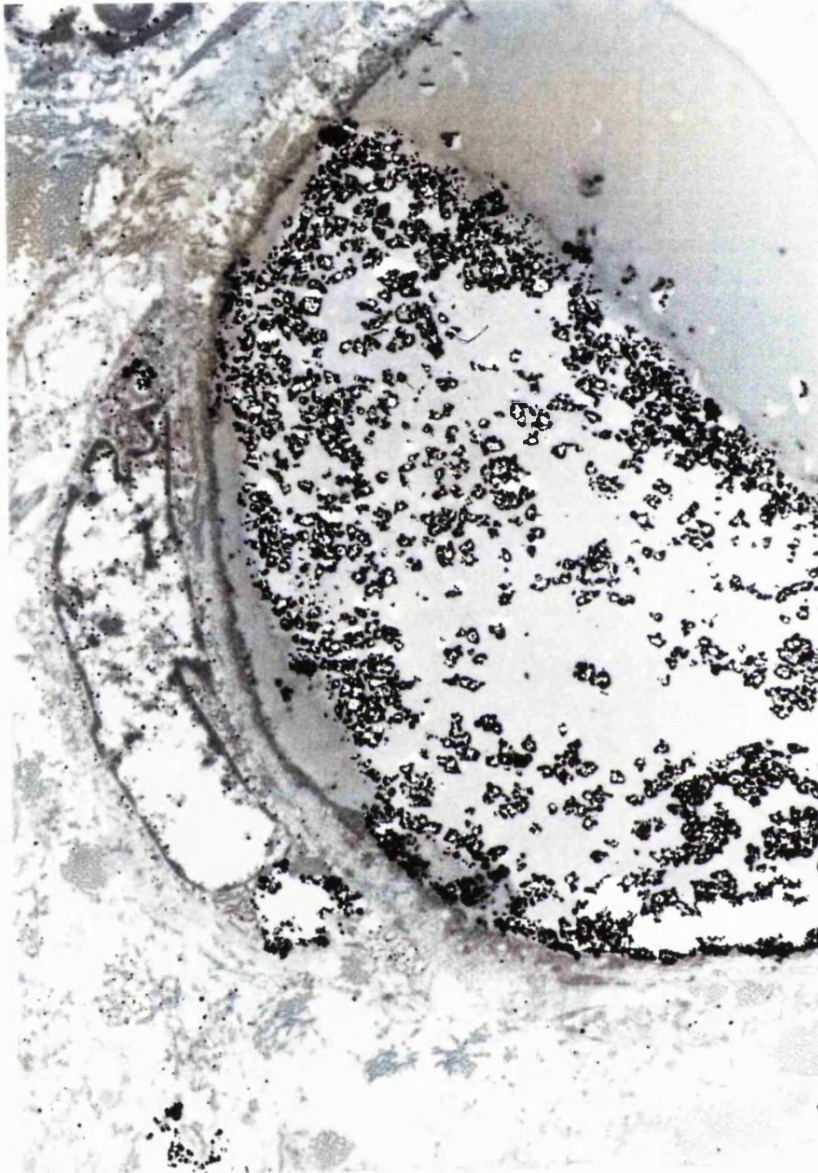
Figure 4.13: Transmission electron micrograph of HCC in explant liver,
perfused with Lipiodol <30 minutes after removal,
and processed after silver nitrate impregnation.
Lipiodol visualised as black droplets in the lumen of a tumour vessel and
within an endothelial cell (magnification x 9500)

Figure 4.14: Transmission electron micrograph of HCC in explant liver,
perfused with Lipiodol <30 minutes after removal,
and processed after silver nitrate impregnation.
Lipiodol visualised as black droplets in the lumen of a tumour vessel and
within an endothelial cell (magnification x 20500)
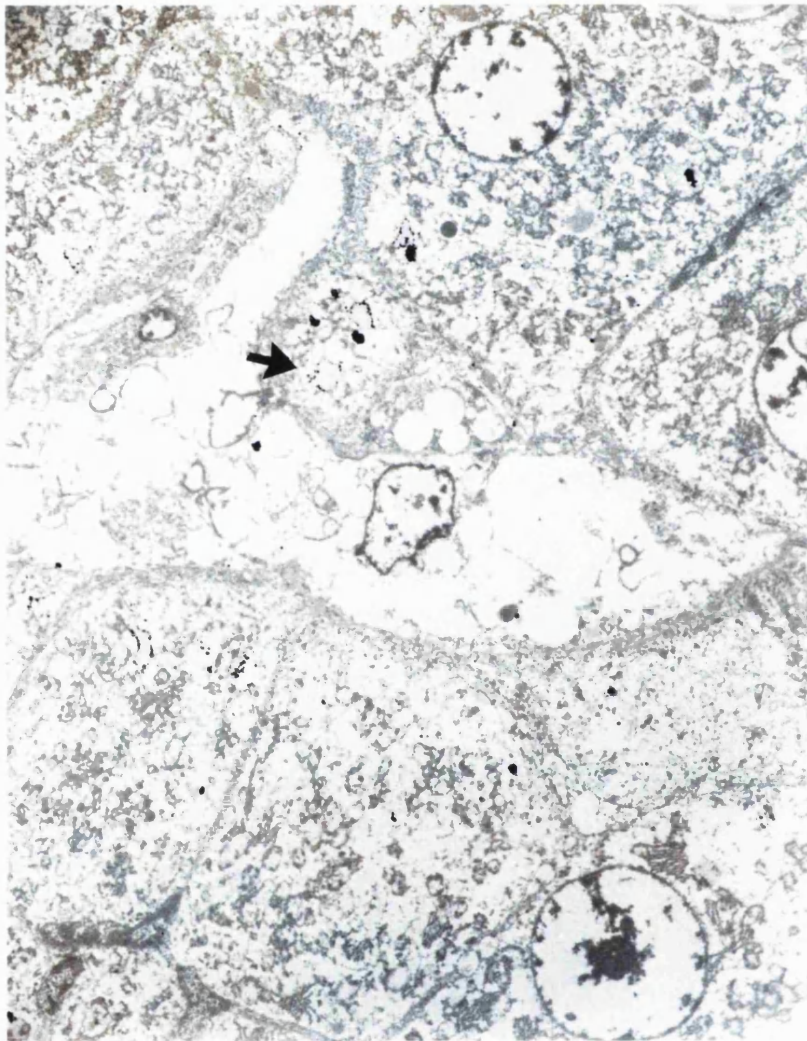
Figure 4.15: Transmission electron micrograph of HCC in explant liver,
perfused with Lipiodol <30 minutes after removal,
and processed after silver nitrate impregnation.
Lipiodol visualised as black droplets within cytoplasmic vesicles
in the tumour cells (magnification x 3000)
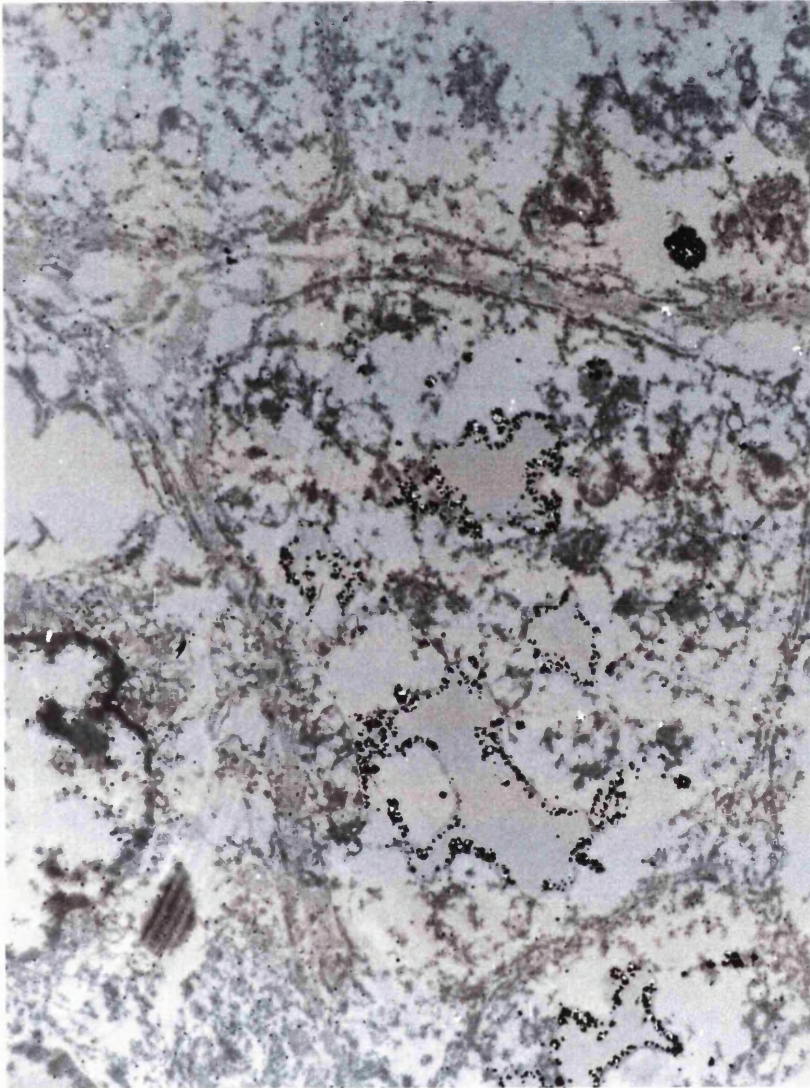
Figure 4.13: Transmission electron micrograph of HCC in explant liver,
perfused with Lipiodol <30 minutes after removal,
and processed after silver nitrate impregnation.
Lipiodol visualised as black droplets in cytoplasmic vesicles
within tumour cells (magnification x 9500)
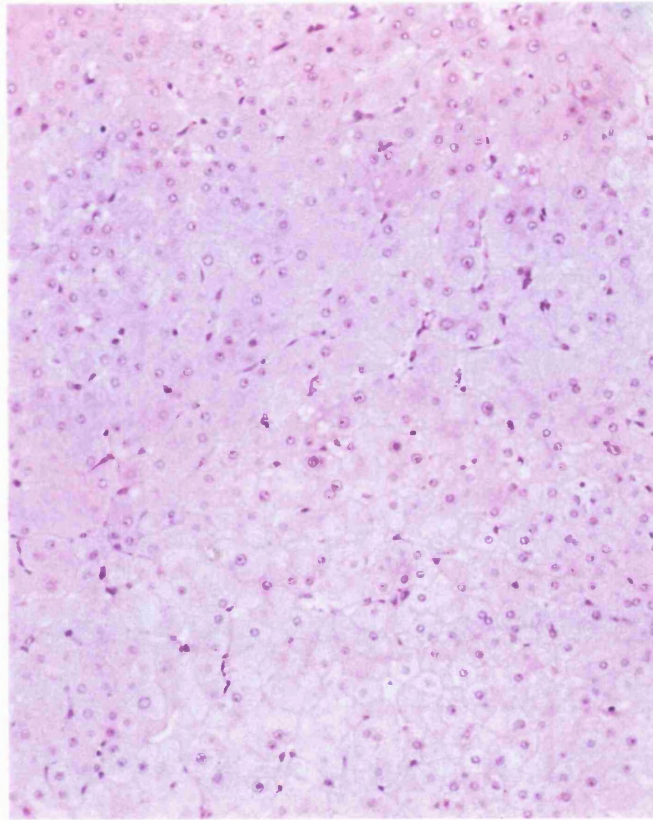
Figure 4.17: Histologic appearance of the non-tumour parenchyma
in an explant liver, following silver nitrate impregnation,
confirms absence of Lipiodol uptake in normal hepatocytes.
Lipiodol   administered ex vivo.
(Haematoxylin & eosin; magnification x 150)

cells in this specimen strongly suggests that Lipiodol incorporation by tumour cells *in vivo* is a relatively rapid and probably active process that occurs within a period of 30 minutes.

4.5   DISCUSSION

Why is Lipiodol selectively retained by HCCs?

The mechanisms of Lipiodol retention in tumours are probably multifactorial, synergistic and cumulative.

Following hepatic arterial injection of Lipiodol, the concentration of Lipiodol droplets is much higher in the tumour vessels than in the vessels of the normal parenchyma. This is confirmed by the histologic findings from this study. The exact reasons for this remain unclear, though physical characteristics of the tumour vasculature may play a significant role; possible explanations have been discussed in Chapter 2.

The tumour cells and the endothelial cells lining tumour vessels then incorporate the lipid. This process may well occur within 30 minutes of Lipiodol injection, as demonstrated in one of the explant livers. In contrast, hepatocytes and sinusoidal endothelial cells in normal liver parenchyma do not take up Lipiodol.

The *in vitro* studies indicate that endothelial cells incorporate Lipiodol rapidly at first, but the intracellular levels of Lipiodol then decrease, suggesting that the cells proceed to metabolise or excrete the oil. On the other hand, tumour cells progressively accumulate Lipiodol. If these differences in behaviour translate into the *in vivo* situation, then they may form the basis of a synergistic mechanism wherein the endothelial cells take up the oil from the vessel lumen and excrete it into the extracellular space, where the tumour cells then incorporate it. Atypical cells which structurally and immunohistochemically resemble Ito (fat storing) cells are also known to be present in the perisinusoidal stroma of HCCs (159, 161). Their potential role in the uptake and retention of lipids such as Lipiodol remains to be clarified.

The prolonged retention of Lipiodol in tumour cells may be related to a morphologic feature of HCCs. In certain situations when normal hepatocytes do take up Lipiodol, there is evidence to suggest that they metabolise it by secreting the lipid moiety into the bile, while the iodine gets excreted in the urine (85, 88, 89). However, HCCs are characterised histologically by the *absence* of bile ducts and portal triads (23, 183-185). Therefore, though tumour cells incorporate Lipiodol when exposed to it, there are no biliary channels for them to excrete it into. This may be the principal reason for the prolonged retention of Lipiodol in HCCs.

The significance of Lipiodol incorporation by tumour and endothelial cells

Evidence for the active incorporation of Lipiodol by tumour cells may have major therapeutic implications. These observations have a significant bearing on the extent to which Lipiodol-targeted therapies may be expected to penetrate into the tumour parenchyma, and the degree of cell-kill that may be achieved. Further studies are indicated to evaluate Lipiodol and other lipids as vehicles for cytotoxic agents and radioisotopes. If entry into tumour cells can be ensured, then ^{125}I may be preferable to ^{131}I as a Lipiodol conjugate. Also, gamma linoleic acid has been shown in *in vitro* studies to enhance the effect of anti-cancer treatments (ref. chapter 6), and linoleic acid is a major component of Lipiodol. It would be important to confirm if the phenomenon is unique to HCCs, or applies to a broad spectrum of human tumour cells.

The incorporation of Lipiodol by endothelial cells lining the tumour vessels suggests that vascular injury may be a mechanism by which Lipiodol-targeted therapies achieve tumour necrosis, and raises intriguing therapeutic possibilities. Attempts at targeting the tumour endothelium have so far largely concentrated on the use of antibodies raised against endothelial surface antigens. The retention of Lipiodol by endothelial cells in HCCs suggests a potential role for it in targeting destructive agents to tumour endothelium.

The role of Lipiodol CT prior to liver transplantation

The findings also suggest that Lipiodol angiography performed prior to liver transplantation may help identify more incidental HCCs in the recipients. The addition of Lipiodol CT to the routine pre-operative investigation of OLT recipients has not been reported so far. However, hepatic angiography is performed routinely in these patients, and injection of Lipiodol can easily be performed at the time of angiography without adding significantly to the morbidity of the procedure.

Patients undergoing transplantation for chronic liver disease who happen to harbour incidental tumours carry a better prognosis than patients who receive OLT primarily for unresectable HCCs (41). It has been suggested that in this group of patients the nature of the underlying liver disease is the main prognostic indicator, and the presence of small occult HCCs has no bearing on the outcome (186). Thus, the diagnosis of an incidental HCC in a given patient may not - in a majority of cases - change the overall plan to proceed to OLT. But it would certainly allow consideration of adjuvant treatment to further improve the result.

Correlation with Lipiodol CT films would also enable the histopathologist studying the explant liver to concentrate on 'suspicious' nodules that have taken up Lipiodol, and increase the number of occult HCCs diagnosed at histology (187). This would allow identification of small HCCs, and possibly lead to a better understanding of morphologic features of the early stages of the neoplastic process.

Chapter 5

LIPIODOL-TARGETED CHEMOTHERAPY VERSUS LIPIODOL-TARGETED RADIOTHERAPY IN THE TREATMENT OF UNRESECTABLE HEPATOCELLULAR CARCINOMA

5.1 INTRODUCTION

Sufficient data now exist to indicate that patients with advanced HCC may be given intra-arterial Lipiodol-targeted therapies with a reasonable degree of safety, while achieving better palliation than systemic or intra-arterial chemotherapy and external beam radiotherapy (29, 44, 48, 114). Drugs that have been conjugated to Lipiodol include 5-fluorouracil (117), doxorubicin (114, 117), epirubicin (119, 120), cisplatin (121), and SMANCS (99). The principal radioisotope used in conjugation with Lipiodol is ^{131}I (131). Comparisons have been made between different chemotherapeutic regimens (ref. chapter 2), but to date there are no reports comparing Lipiodol-targeted chemotherapy with Lipiodol-targeted radiotherapy. This study attempts to assess the benefits of Lipiodol-targeted epirubicin chemotherapy and Lipiodol-targeted ^{131}I radiotherapy in two comparable groups of patients at the same centre.

MATERIALS AND METHODS

Patient selection

From October 1988 to June 1993, all patients presenting with unresectable HCC at the University Departments of Surgery and Medicine, Royal Free Hospital were considered for inclusion in the study. Initial investigations for each patient included haematology, liver biochemistry, coagulation profile, viral hepatitis serology and serum alpha fetoprotein. The lesions were staged in every instance by abdominal ultrasound and CT, chest X-ray and CT, and a radioisotope bone scan (Table 5.1). Confirmation of the diagnosis was attempted by Tru-cut biopsy or needle aspiration cytology. On completion of the staging investigations, patients were invited to join the study only if they met the requirements listed in Table 5.2. Patients with evidence of extrahepatic spread of tumour within the abdomen, or with distant metastases were excluded from the study. Resectability was determined at a joint consultation between physicians and surgeons. Patients with resectable lesions who declined surgery were not included. Very large tumour size, multifocal lesions involving both lobes, involvement of major vascular structures in or around the liver, extrahepatic tumour

Table 5.1

Investigations prior to consideration for inclusion in the trial of Lipiodol targeted therapies for unresectable HCC

I. *Haematology, biochemistry and viral serology*

- Full blood count

- Blood urea; Serum electrolytes & creatinine

- Liver function tests; Serum albumin; Coagulation profile

- Serum Alpha fetoprotein

- Serum markers for Hepatitis B & C viruses

II. *Staging investigations*

- Chest x-ray

- Abdominal ultrasound

- Abdominal CT scan

- Chest CT scan

- Bone scan

III. *Histologic confirmation*

- Liver biopsy (Trucut/Fine needle aspiration cytology)

spread, and poor hepatic reserve due to concomitant cirrhosis were the common reasons why the tumours were unresectable. Abnormal liver function *per se* was not a contraindication, and patients included in the study ranged through Child-Pugh grades A to C (188). Patients in fulminant liver failure with uncorrected coagulopathy were not included; they were reconsidered at a later date if their liver function improved. Coagulation abnormalities were corrected as far as possible to reduce the risks of angiography. Obstruction of the main trunk of the portal vein, demonstrated on angiography or ultrasonography, was considered a contraindication for targeted arterial therapies. Only patients with a WHO performance status of 0-3 were invited to participate in the study. Advanced age was not deemed to be a contraindication if the patient's general physical condition was satisfactory, with no serious concurrent medical problems. Tumour size, number or stage did not influence patient selection. Written informed consent was obtained from all patients who agreed to participate in the study.

Patient numbers and characteristics

A total of 95 patients participated in the study - 69 received Lipiodol-Epirubicin and 26 received Lipiodol ^{131}I. Their age and sex, the nature of their underlying liver disease, and their tumour stages are shown in Table 5.3. The first 67 patients in the series were allocated to one arm or the other according to treatment availability and patient preference. On several occasions consecutive patients were administered Lipiodol-Epirubicin because ^{131}I was not available. The last 28 were prospectively randomised to receive one treatment or the other: 17 received Epirubicin and 11 received ^{131}I. Choice of treatment for each of these patients was determined by drawing a computer-generated randomisation slip from a sealed envelope. The characteristics of this subset of patients are shown in Table 5.4. Histologic confirmation of the diagnosis was available in 63 of the 69 Epirubicin recipients (15 out of 17 in the randomised trial group) and in 23 out of 26 patients who received ^{131}I (10 out of 11 in the randomised trial group). The rest were diagnosed as having HCC on the basis of tumour circulation visualised at angiography, and a raised AFP level. Of the 69 Epirubicin recipients, 52 were Caucasian and 17 were of Asian or African origin. In the ^{131}I group,

Table 5.2

**Criteria for inclusion in the trial
of Lipiodol targeted therapies for unresectable HCC**

(1)   Tumour confined to the liver

(2)   Tumour unresectable

(3)   Absence of fulminant liver failure or coagulopathy

(4)   Portal vein patency

(5)   Physical condition: WHO performance status of 0-3

Table 5.3

95 patients treated with Lipiodol-Epirubicin or Lipiodol ^{131}I:
Demographic characteristics, nature and severity of underlying liver
disease, and tumour stages

	Lipiodol-Epirubicin	Lipiodol ^{131}I
Total patients	69	26
Sex	54 male, 15 female	22 male, 4 female
Age	20-81 years Median age 61 years	20-75 years Median age 64 years
Cirrhotics Child-Pugh grades	61 A 27, B 27, C 7	18 A 13, B 5, C 0
Aetiology of cirrhosis: Hepatitis B virus Hepatitis C virus Alcohol Cryptogenic cirrhosis Other	 23 8 14 9 8	 9 4 4 1 0
Okuda Tumour Stage	I 14, II 37, III 18	I 6, II 19, III 1

Table 5.4

28 patients treated with Lipiodol-Epirubicin or Lipiodol ^{131}I
in a prospective randomised trial:
Demographic characteristics, nature and severity of liver disease,
and tumour stages

	Lipiodol-Epirubicin	Lipiodol ^{131}I
Total patients	17	11
Sex	13 male, 4 female	9 male, 2 female
Age	35-73 years Median age 62 years	20-75 years Median age 63 years
Ethnic break-up: Caucasian Middle East Far East	 6 8 3	 7 4 0
Cirrhotics Child-Pugh grades	16 A 8, B 8, C 0	6 A 5, B 1, C 0
Aetiology of cirrhosis: Hepatitis B virus Hepatitis C virus Alcohol Cryptogenic cirrhosis Other	 5 4 4 2 1	 1 4 1 0 0
Okuda Tumour Stage	I 6, II 7, III 4	I 2, II 8, III 1

21 out of 26 were Caucasian and the rest of Asian origin. The detailed ethnic break-up of the two randomised groups has been shown in Table 5.3. Seventy-nine of the 95 patients had underlying cirrhosis, and the severity of their disease was graded according to Pugh's modification of Child's criteria (188). Two patients had recurrent HCC following previous resection (1 received Epirubicin; the other received ^{131}I in the randomised trial). Fifteen of the 28 patients in the randomised study had multifocal lesions on CT scans. All tumours were staged according to Okuda's staging criteria. Of the 28 patients in the randomised trial, 23 were symptomatic at presentation. Symptoms included abdominal pain or discomfort (12), weight loss (8), anorexia and nausea (10), presence of a palpable abdominal lump (4), fever (2), lassitude (4), ascites (3) and jaundice (2). All patients gave written informed consent to their inclusion in the study, and the study protocol was approved by the Royal Free Hospital Ethical Committee. Prior to the commencement of the prospective randomised study, a proportion of the patients were followed up by a colleague (Mr J R Novell), and he has kindly agreed to the inclusion of some data that have already been reported by him (120).

Administration of treatment

Lipiodol-epirubicin and Lipiodol-^{131}I were administered at hepatic angiography. Dosage of epirubicin was calculated at 75 mg/m^2. In the event of impaired liver function, the dose was reduced by 25% if the serum bilirubin was > 30 μmol/l, and by 50% if the serum bilirubin was > 100 μmol/l. The desired amount of epirubicin (4'-epi-doxorubicin available as Pharmorubicin, Farmitalia Carlo Erba) was dissolved in 5-10 ml of sodium meglumine diatrizoate (Urografin 290, Schering AG) and added to 10 ml Lipiodol Ultra Fluid. A stable colloidal emulsion of epirubicin and Lipiodol was created by agitation in a Pulsatron ultrasonic agitator for 5 minutes. These procedures were carried out with aseptic precautions in the hospital's Manufacturing Pharmacy unit, less than 6 hours prior to injection. The preparation was further agitated vigorously prior to injection. Doses administered ranged from 40 to 120 mg.

Patients scheduled to receive Lipiodol-^{131}I underwent prior ^{99}Tc colloid liver scintigraphy, to allow comparison with subsequent scintiscans for

^{131}I uptake. Lipiodol-^{131}I (CIS Bioindustries, France) was supplied for each individual patient on order, as a 2 ml vial with an activity of 15 - 40 mCi (550 - 1480 MBq). This was diluted to a total volume of 12 ml with ordinary Lipiodol, using two sterile 20 ml syringes and a luer lock 3-way tap. Tumour size was not a consideration in determining dosage in either group. No pre-medication with potassium iodide was necessary as the contrast media administered during prior screening investigations provided adequate thyroid blockade. Dosage administered at a single session ranged from 220 to 1315 MBq (median 820 MBq).

At angiography, the hepatic artery was cannulated under fluoroscopic control via the transfemoral approach, using the Seldinger technique. The drug/isotope was injected over a period of one minute, and the line flushed with 20 ml of normal saline after injection. Appropriate radiation protection measures were taken by the medical staff.

Follow-up and assessment

Patients were closely observed in the first 12 hours following treatment, particularly to exclude groin haematoma or vascular injury. Thereafter, they were assessed daily to chart the progression of their symptoms and clinical condition. Their full blood count, serum electrolytes, blood urea, serum creatinine, liver function tests, serum albumin and coagulation profile were checked on alternate days during the hospital stay. A CT scan of the liver was performed in all cases at 7 to 10 days after treatment, to assess Lipiodol retention in the tumours (Figure 5.1 A).

Lipiodol-^{131}I recipients were nursed and monitored in a radiation shielded room for the first 48-72 hours after treatment. Contact of patients with medical staff and family members was kept to a minimum. Patients were transferred to ordinary wards or discharged into the community only after the residual activity was measured at <800 MBq, as per the recommendations of the British National Radiological Protection Board (189). In addition to the post-treatment CT scan, all Lipiodol-^{131}I recipients underwent gamma scintigraphy or a liver SPECT (single photon emission computerised tomography) 24-48 hours after treatment, to assess uptake of radioactivity by the tumour. This was repeated once again within the next 20 days. Gamma

143

scintigraphy was carried out with a Scintronix 480S Digicamera fitted with a high energy collimator. Planar anterior and posterior images of the liver and thorax were obtained for analysis (Figure 1.3). On these images, regions of interest were drawn around the tumour, the liver and the lungs, and total activity measured in each region. Six patients underwent SPECT, which provides three dimensional data and allows exclusion of activity from the surrounding tissues (Figure 5.2). These scans were carried out on an IGE Gemini 700 camera fitted with a 400 KeV parallel-hole high resolution collimator, linked to a Saturn Nuclear Medicine computer for data storage and image analysis. IGE software was used for image reconstruction and dosimetry. The post-therapy scintiscan images were also compared with pre-treatment ^{99}Tc colloid scan images to assess if ^{131}I uptake had occurred through the entire tumour.

Patients were seen in the outpatient clinic 8 weeks after their treatment, with a fresh CT scan, and at similar intervals thereafter. Tumour sizes (maximum tumour diameter) were compared to pre-treatment scans to assess response (Figure 5.1 B). Tumour response, as determined by two observations 8 weeks apart, was graded as follows: *(1) Complete Response (CR)*: Disappearance of all known disease *(2) Partial Response (PR)*: 50% or more decrease in tumour load; no appearance of new lesions or progression of any lesion *(3) No Change (NC)*: Less than 50% decrease in total tumour load; no progression of any lesion *(4) Progressive Disease (PD)*: Increase in size of one or more lesions or appearance of new lesions. To obtain a simple measure of the palliative effect achieved, patients on the randomised controlled study were asked at follow up appointments to grade the symptomatic relief accorded by the treatment as "Good", "Moderate" or "Poor".

Further sessions of the same therapy were offered if appreciable tumour response or symptomatic relief had been obtained from the first session, the treatment had been well tolerated, and the patient's overall condition and liver function were judged satisfactory. A total of 133 treatments were administered to the 69 epirubicin recipients (range 1 - 4; mean treatment sessions per patient: 1.9). The 26 ^{131}I recipients received 43 treatments (range 1 - 3; mean 1.7). Within the randomised trial, the break-up of treatment sessions was as shown in Table 5.5.
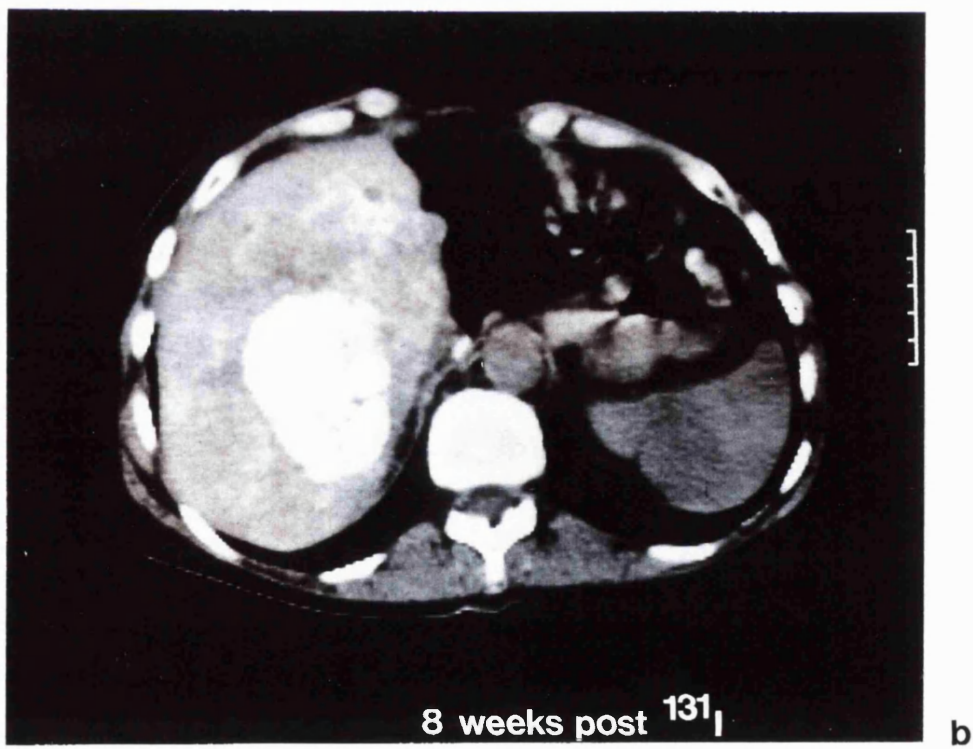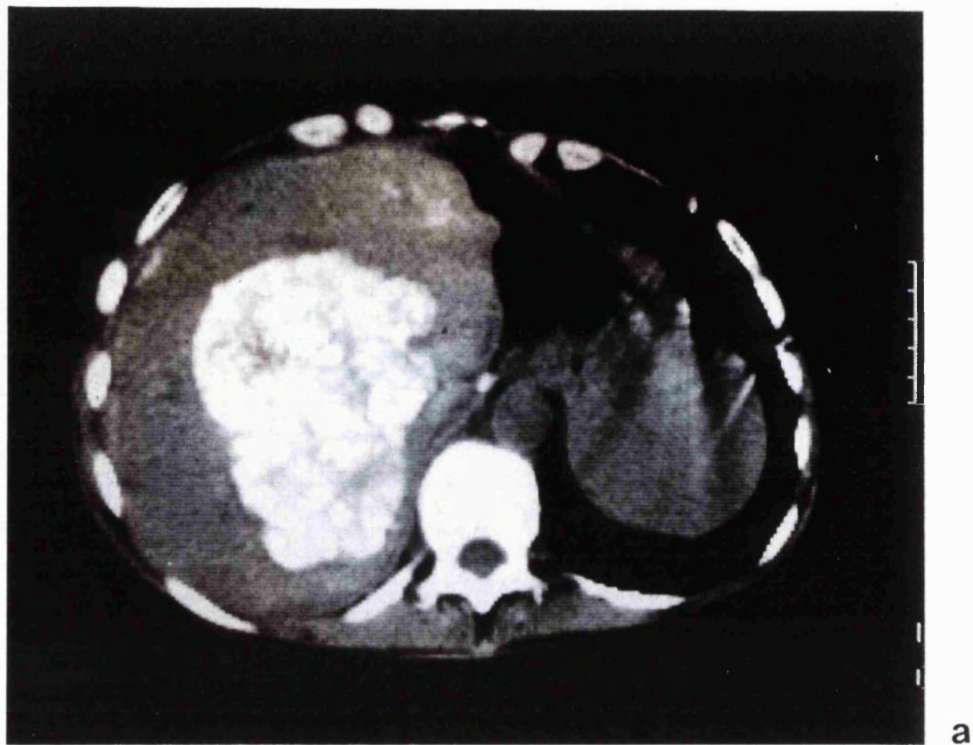
Figure 5.1: CT scan of liver with HCC, (A) before and (B) after Lipiodol ^{131}I radiotherapy, demonstrating reduction in tumour size
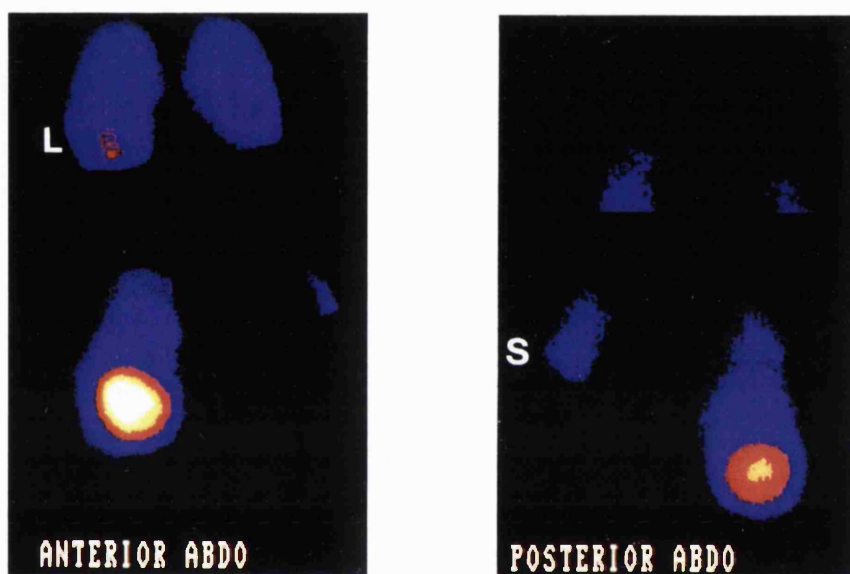
Figure 5.2: Anterior and posterior views on a liver SPECT
48 hours after hepatic arterial administration of Lipiodol ^{131}I for treatment of a HCC.
Bright area indicates uptake of activity by the tumour. Low levels of
activity seen in the rest of the liver, the lungs (L) and the spleen (S)

Table 5.5

Total number of treatment sessions administered in the
two treatment arms of the prospective randomised study

	Epirubicin	^{131}I
One treatment	10 patients	8 patients
Two treatments	5 patients	2 patients
Three treatments	2 patients	1 patient
Mean sessions per patient	1.53	1.36

Table 5.6

Tumour responses achieved by the two treatment modalities
(Figures in parentheses represent patients in the
prospectively randomised groups)

	Epirubicin	^{131}I
Complete response	0 (0)	0 (0)
Partial response	3 (3)	7 (2)
No change	18 (4)	8 (4)
Progressive disease	17 (6)	7 (3)
Total	38 (11)	22 (9)

Survival of patients was calculated upto either (1) death (2) loss to follow up (3) administration of other treatments, or (4) conclusion of the study in December 1993.

Statistical analysis was carried out on a SUN mainframe computer using the software programme SAS (Statistical Analysis System) Version 5 (© SAS Institute Inc., Cary, North Carolina, USA; 1985).

RESULTS

Effectiveness of localisation

Assessment on CT scans

Sixty-six of 69 epirubicin recipients underwent a CT scan 7-10 days after therapy. All patients showed selective localisation of Lipiodol in the tumour(s), compared to the rest of the liver. All images were assessed by the same consultant radiologist, and the degree of Lipiodol retention by the tumour was judged to be satisfactory in every patient.

Radiation dosimetry

Uptake of Lipiodol ^{131}I, as assessed on gamma camera imaging or on SPECT, revealed selective localisation in all cases. 'Area of interest' calculations in 15 patients revealed a mean tumour: liver ratio of 9:1. The mean ($\pm$ standard deviation) cumulative radiation dose to the tumour was calculated at 34.7 ($\pm$32.4) Gy. Mean cumulative doses to non-tumour liver parenchyma and to the lungs were 3.3 ($\pm$1.5) Gy and 4.4 ($\pm$2.3) Gy respectively.

Tumour response

Diminution in size

The changes in tumour size following treatment are documented in Table 5.6. The principal columns of figures represent the total numbers of patients while the figures in parentheses represent patients in the

randomised study. Overall, 21 of 38 (55%) evaluable epirubicin recipients and 15 out of 22 (68%) [131]I recipients demonstrated a slowing of their disease process (PR or NC). Within the randomised study, the relative proportions were similar - 7/11 (64%) with epirubicin and 6/9 (67%) with [131]I.

AFP levels

Amongst patients receiving epirubicin, 43 had a raised serum AFP level prior to receiving therapy. Persistent fall in AFP was documented in 4. In the ^{131}I group, 22 had a raised serum AFP at presentation, and a fall was documented in 2 patients.

Palliation of symptoms

In the prospective randomised groups 14/17 epirubicin recipients and 9/11 ^{131}I recipients were symptomatic at presentation. The palliative effects achieved were graded by the patients themselves as follows: Epirubicin - 'good' 8, 'moderate' 2, 'poor' 3, and 1 was difficult to assess; [131]I - 'good' 5, 'moderate' 1, 'poor' 3. Thus 10/14 patients treated with epirubicin and 6/9 patients treated with ^{131}I felt they had received good or moderate palliation of symptoms.

Treatment-related morbidity and mortality

Fuminant hepatic failure was the principal cause of death within 30 days of either form of treatment. Seven of 69 epirubicin recipients developed this complication, with 5 deaths. In the ^{131}I group, 4 out of 26 patients developed this complication and died from it. In the epirubicin group, there were 3 other mortalities, 2 from sudden tumour rupture and one from a perforated peptic ulcer. The overall 30-day mortality was 12% with epirubicin and 15% with [131]I. It should be clarified that 6 of these 12 deaths occured after the *second* session of treatment. All but one patient had cirrhosis of Child-Pugh grade B or C and Stage II or III tumour. In some of the patients, it was difficult to determine whether the liver failure had been precipitated by the treatment or by tumour progression and underlying cirrhosis.

Table 5.7

Morbidity related to treatment of unresectable HCCs
with Lipiodol epirubicin and Lipiodol ^{131}I.
Figures in brackets [ ] represent data from
the prospectively randomised groups

	Lipiodol epirubicin n = 69 [n = 17]	Lipiodol ^{131}I n = 26 [n = 11]
Pyrexia	53 (84%) [15 (88%)]	10 (38%) [3 (27%)]
Raised serum bilirubin/ transaminases	29 (42%) [3 (18%)]	7 (27%) [2 (18%)]
Anorexia, nausea or vomiting	8 (12%) [4 (23%)]	1 (4%) [1 (9%)]
Abdominal pain	6 (9%) [2 (12%)]	1 (4%) [1 (9%)]
Anaemia	19 (28%) [1]	0
Thrombocytopenia	15 (22%) [0]	0
Leucopenia	13 (19%) [3 (18%)]	0
Chest infection	4 [4 (23%)]	2
Alopecia	4 [1]	0
Ascites	2 [2]	1
Peptic ulceration	2 [2]	0
Variceal bleeding	1 [1]	0

150

Technical complications arising from the angiographic procedure were rare. One patient developed hepatic arterial dissection following cannulation. The procedure was abandoned but repeated successfully 2 weeks later.

The other complications arising from the two treatments are listed in Table 5.7. Statistical comparison of the incidence of complications in the two groups (Chi square test with Yates' correction or Fisher's exact test if the numbers were small) revealed that pyrexia was significantly more common in the patients receiving epirubicin ($p = 0.001$ for the entire cohort and $p = 0.003$ for the randomised subset). Pyrexia is known to occur after systemic epirubicin therapy, and is probably a consequence of tumour necrosis. Amongst the non-randomised patients, there was also a significantly higher incidence of anaemia ($p = 0.007$), leucopenia ($p = 0.017$) and thrombocytopenia ($p = 0.009$) following epirubicin therapy. Howver, these complications related to bone marrow suppression were generally transient and self-limited. Two patients with leucopenia required supportive therapy. With either therapy, a rise in serum bilirubin and transaminases which returned to normal within 10 days was frequently noted. Some patients suffered an aching discomfort in the right hypochondrium, probably secondary to tumour necrosis. The chest infections recorded in some instances may have been pneumonitis secondary to localisation of small amounts of Lipiodol in the lungs. The alopecia was partial and reversible.

Survival

Of 69 Lipiodol-Epirubicin recipients, 13 (5 Stage I, 8 Stage II) were excluded from survival analysis (2 were lost to follow-up within 2 months of receiving therapy, 4 underwent hepatic resection, 3 underwent liver transplantation and 4 received other forms of treatment). Fifty-six patients were evaluated. Fifty-three were followed up for a minimum period of 1 year (or to death), and the last three were alive at 6, 6 and 8 months after therapy. At the conclusion of the study, 49 of the 56 were dead, 46 from tumour-progression, and 3 from unrelated causes. Seven patients were alive at the end point of the study having survived 6, 6, 8, 12, 14, 21 and 36 months.

Of 26 Lipiodol-^{131}I recipients, one was lost to follow-up soon after treatment and was excluded from the analysis. Twenty-five patients were evaluated - 20 were followed up for at least 1 year (or to death), 4 were followed up for 6 months and one was lost to follow-up after 2 months. Seventeen of the 25 evaluated were dead at the end of the study, all from tumour progression. Six were alive (6, 6, 6, 6, 12 and 19 month survivals), and 2 had been lost to follow-up at 2 and 16 months respectively.

The actuarial survival according to tumour stage in the two treatment groups is depicted in Figures 5.3 and 5.4. Survival at 6, 12 and 24 mths was 40%, 25% and 6% with Epirubicin, and 58%, 25% and 0% with ^{131}I. Kaplan-Meier curves for the two subsets of patients in the prospective randomised trial are depicted in Figure 5.5. No statistically significant differences in survival were found between the two groups, either in the overall study or in the prospectively randomised trial.

Prognostic factors influencing survival

The influence of age, severity of underlying cirrhosis (Child-Pugh grades A, B or C), tumour stage (Okuda I, II or III), and degree of tumour response at 2 months (PR or NC or PD) on survival was assessed using the Cox proportional hazards model. Univariate analysis revealed significant associations between survival and the Child-Pugh grade of cirrhosis (risk ratio = 1.41 for every increase in grade of cirrhosis; p = 0.012), the Okuda tumour stage (risk ratio = 2.79 for every increase in tumour stage; p = 0.0001) and the degree of tumour response (risk ratio = 2.75 for every decrease in the degree of response; p = 0.0002). Multivariate analysis revealed that grade of cirrhosis did not contribute significantly to the prognostic information after allowing for tumour stage, but tumour stage had a strong prognostic significance (risk ratio = 2.15 for every increase in tumour stage; p = 0.027). The degree of tumour response as judged on a CT scan at 2 months correlated significantly with further survival (risk ratio = 3.35 for every decrease in the degree of response; p = 0.0001).

No significant association was found between the administered dosage of therapeutic agent (mg of epirubicin or MBq of ^{131}I) and tumour
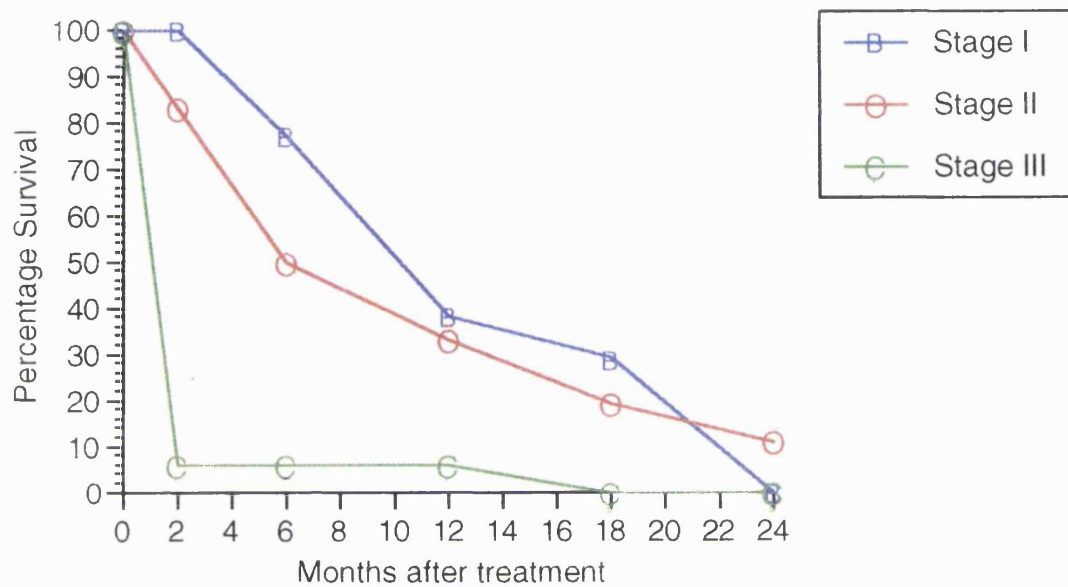
Unresectable Hepatocellular Carcinoma treated with Lipiodol Epirubicin
Total patients: 56 (Okuda Stage I 9, Stage II 29, Stage III 18)

Figure 5.3: Survival following treatment of unresectable HCC
with Lipiodol-epirubicin (n = 56)
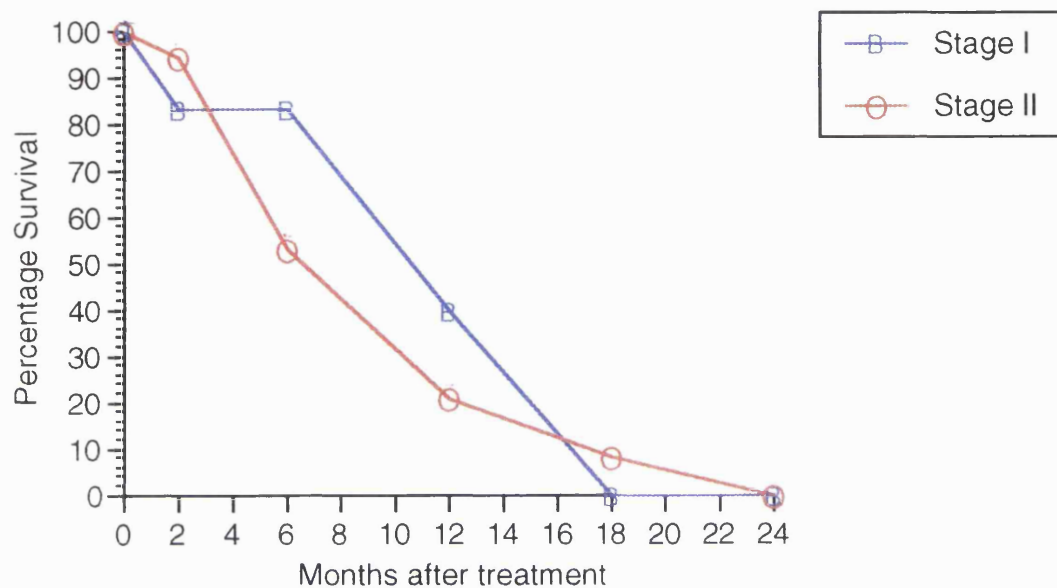
Unresectable Hepatocellular Carcinoma treated with Lipiodol Iodine 131
Total patients: 25 (Okuda Stage I 6, Stage II 18, Stage III 1)
The patient with Stage III disease died within 2 months of therapy

Figure 5.3: Survival following treatment of unresectable HCC
with Lipiodol ^{131}I (n = 25)
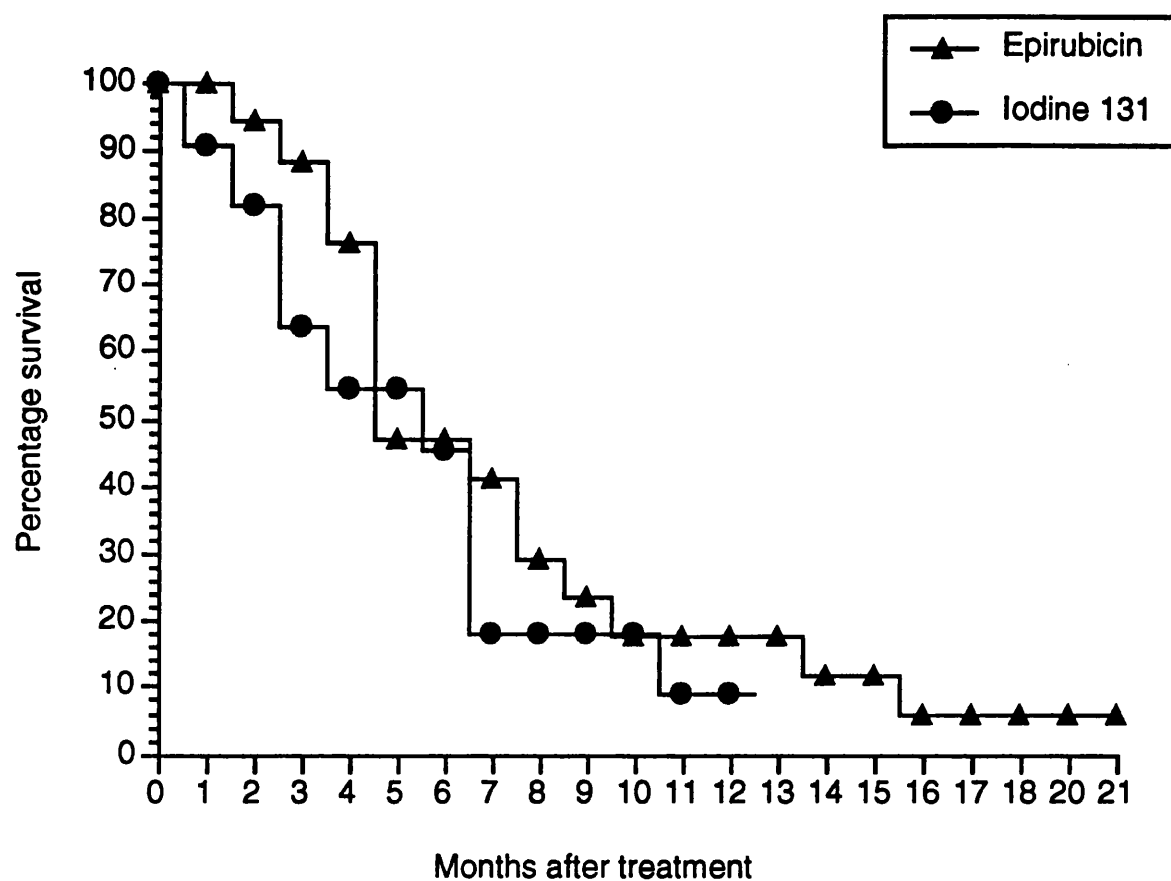
Figure 5.5: Kaplan-Meier curves depicting survival
following treatment of unresectable HCC with Lipiodol epirubicin
or Lipiodol ^{131}I in a prospective randomised trial

155

response. However, among ^{131}I recipients, there was an association between tumour response and the radiation dose to the tumour. Patients with PD had a significantly lower mean tumour radiation dose than those with NC or PR ($p < 0.05$; pooled estimate of variance).

DISCUSSION

The majority of reports on the use of Lipiodol-targeted therapies have come from the Far East. The European experience provides a useful counterpoint, reflecting a different population of patients, possibly a different natural history of the disease, and at times, a different treatment philosophy. This study involves a large patient cohort treated at a single western centre, and is also one of the first attempts to compare Lipiodol-targeted chemotherapy with Lipiodol-targeted radiotherapy.

Choice of patients, therapeutic agents and dosage

Patients with Okuda Stage III disease suffered a very high incidence of complications with either treatment, without any appreciable improvement in survival. It would be reasonable to conclude that this sub-group of patients should not be offered Lipiodol-targeted intra-arterial therapies.

Of the numerous cytotoxic drugs that have been used in combination with Lipiodol, epirubicin was chosen because of its documented efficacy against HCC and its acceptable range of toxicity. Quantitation of drug localisation in the tumour remains a difficult problem. Assessments of Lipiodol uptake from CT scans have their limitations, and are at best semi-quantitative (105). The available parenteral preparations of epirubicin are lipophobic, which led to initial concerns that the drug may separate from the Lipiodol in vivo. A biodistribution study of radio-labelled doxorubicin administered via the hepatic artery has since confirmed that administration with Lipiodol increases the intratumoral concentration of the drug (84). Nevertheless, development of lipophilic drug formulations is likely to ensure more efficient delivery.

Embolisation was not part of the therapeutic protocol in this study, but

there now is evidence that while it does not add to the morbidity or mortality, embolisation of the tumour immediately following the injection of the drug-Lipiodol conjugate may enhance intratumoral drug concentrations (84) and add to the survival benefit (94, 95).

Amongst available radioisotopes, ^{131}I can be conjugated to Lipiodol with relative ease. As it is a beta and gamma emitter, its localisation can be accurately assessed by gamma scintigraphy, while the beta radiation exerts the therapeutic effect. The doses of radiation used in this study have been conservative, and a preliminary report from another centre suggests that higher doses may be administered safely and more often, and may achieve better responses (190). Dosage may also be calculated on the basis of tumour size, and the extent to which it takes up a tracer dose of isotope (128). There is data to suggest that portal vein occlusion need not necessarily be considered a contraindication for these treatments, especially if they are not combined with embolisation (190).

Tumour responses

The arrest or diminution in tumour size at two months obtained in nearly two-thirds of the patients treated with either modality is encouraging, given the overall grim prognosis of untreated HCC. Tumour response at 2 months also proved to be a good indicator of further survival. Though tumour response in ^{131}I recipients was found to be improved if a higher dose of radiation was retained by the tumour, this did not always correlate with the amount of activity administered (dose/activity ratios ranged from 0.2 to 16.1, with a mean of 4.1 $\pm$ 4.3 SD; tumour radiation dose measured in cGy, administered activity measured in MBq). It was therefore difficult to predict tumour response in a given patient.

AFP levels have been reported to correlate well with tumour progression or regression (119). In this study however, a sustained fall in AFP was recorded only in a very small number of patients, and no correlation could be found between post-treatment AFP levels and tumour response or survival. The reasons for this are not apparent.

Quality of life is a major criterion is determining choice of palliative

therapies. Though the majority of reports on the use of Lipiodol-targeted treatments have commented scantily on this aspect, the existing data suggest that these therapies offer effective palliation (116). Our experience with the patients within the randomised trial indicates that both modalities offer satisfactory palliation of symptoms in a majority of patients.

Survival benefit offered by Lipiodol-targeted therapies

For ethical reasons, it was not possible in this study to have a control group of patients who were not offered any treatment at all. The question of whether these therapies offer a survival benefit can therefore be answered only by comparing with historical controls, despite the flaws inherent in such comparisons. The natural history of HCC differs in different parts of the world, and a close and recent parallel to our clinical situation may be found in the 30 untreated patients reported by Vetter et al (32) from France. Comparison with this group indicated a survival benefit with either treatment in Stage I and Stage II disease at 6 and 12 months. At 6 months 25% of untreated Stage I and 14% of untreated Stage II patients were alive. In this study, with epirubicin therapy the figures were 77% and 50% for Stages I and II respectively, and with ^{131}I the figures were 83% and 53%. At 1 year, for patients with Stage I lesions the survival figures were - untreated 12%, epirubicin 38%, ^{131}I 40%; for Stage II - untreated 0%, epirubicin 33%, ^{131}I 21% (Figures 5.6 and 5.7).

Lipiodol chemotherapy versus Lipiodol radiotherapy

No major differences emerged between the two therapies in terms of palliative effect, survival benefit, and procedure-related mortality. Median hospital stay was 6 days in both groups studied prospectively. Chemotherapy patients had a significantly higher incidence of pyrexia, but this usually responded to Paracetamol, and settled within the first week. Epirubicin also caused marrow suppression and abnormalities in haematologic indices in a significant number of recipients, but this again was transient and generally did not require supportive measures. The incidence of isolated individual complications was higher in the chemotherapy group, though not statistically significant. Radiotherapy

requires greater infrastructure and a dedicated clinical unit. The recipients have to remain in isolation for 48-72 hours, and staff have to adopt appropriate precautions. A comparison of the financial costs involved can be made only after Lipiodol-^{131}I becomes commercially available for unrestricted purchase.

Strategies for improving survival: Prospects for future clinical studies

The poor prognosis associated with an advanced tumour stage at the time of treatment emphasises the need for early diagnosis of these tumours. Regular screening of all cirrhotics with ultrasonography and serum AFP measurements may help detect HCCs at an earlier stage in a large proportion of patients (24), and significantly improve their prognosis.

Further therapeutic trials with larger patient numbers, possibly on a prospective multi-centre basis, may provide more information on the relative merits of these therapies, and also diminish the potential for Type II statistical errors. Apart from epirubicin, good results have been reported with other agents such as cisplatin (121, 191) and doxorubicin (114, 117), and prospective comparisons amongst available regimens would be useful, with particular emphasis on lipophilic formulations. Comparisons are also indicated amongst the available radioisotopes, including Yttrium-90 and Iodine-125. Consideration should be given to administering as high a cumulative radiation dose to the tumour as is safely possible.

As data become available on other relatively new therapeutic options such as alcohol injection (51, 52), laser ablation (55) and cryoprobes (54), the indications for Lipiodol-targeted therapies in unresectable HCC may become better defined in terms of tumour size, number, location and stage. It may be possible to combine Lipiodol-targeted therapies with some of the newer modalities, and further improve the results obtained (115). The use of these treatments may even extend to lesions which at present are treated surgically. In at least one study, transcatheter oily chemo-embolisation has yielded results comparable to resection or transplantation for Okuda Stage I and II HCCs (192). Finally, Lipiodol-

targeted therapies are now being tried as pre-operative adjuncts to resection and orthotopic liver transplantation for HCC, and the long term results of these studies are awaited.

Figure 5.6

100
90
80
70
60
50
40
30
20
10
0

Percentage Survival

0   6   12   18   24
Months after treatment

■— RFH Epirubicin
—O— RFH Iodine 131
▲— Controls (Vetter)
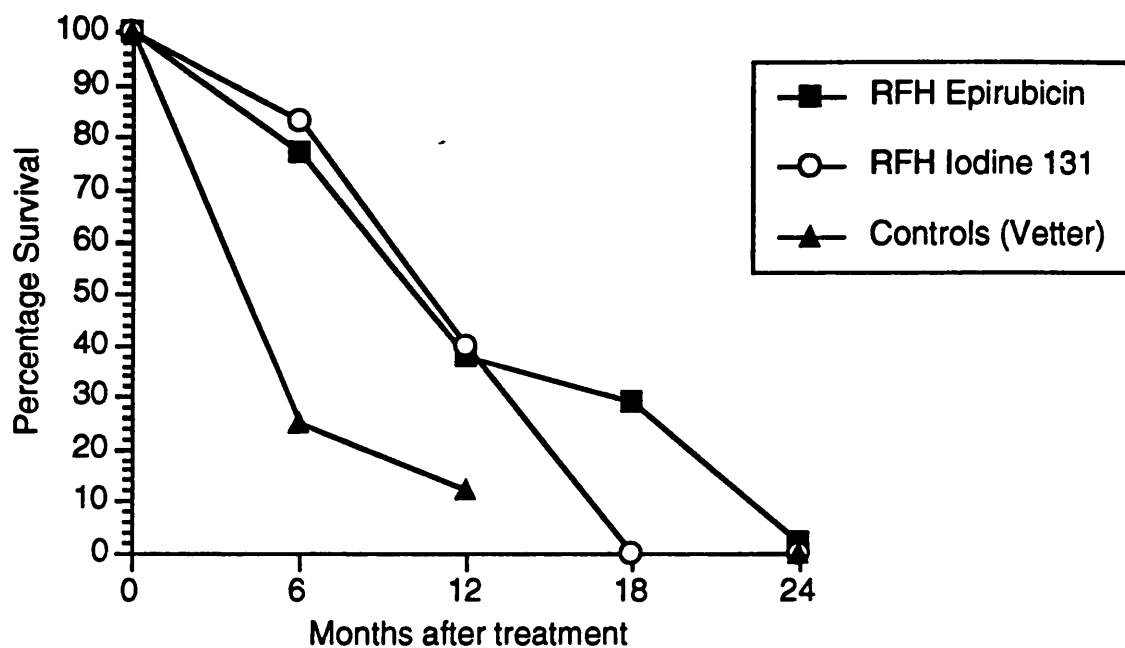
Unresectable Okuda Stage I Hepatocellular Carcinoma
treated with Lipiodol Epirubicin (n=9) or Lipiodol Iodine 131 (n=6),
compared with untreated controls (n=8) reported by Vetter et al.

Figure 5.7
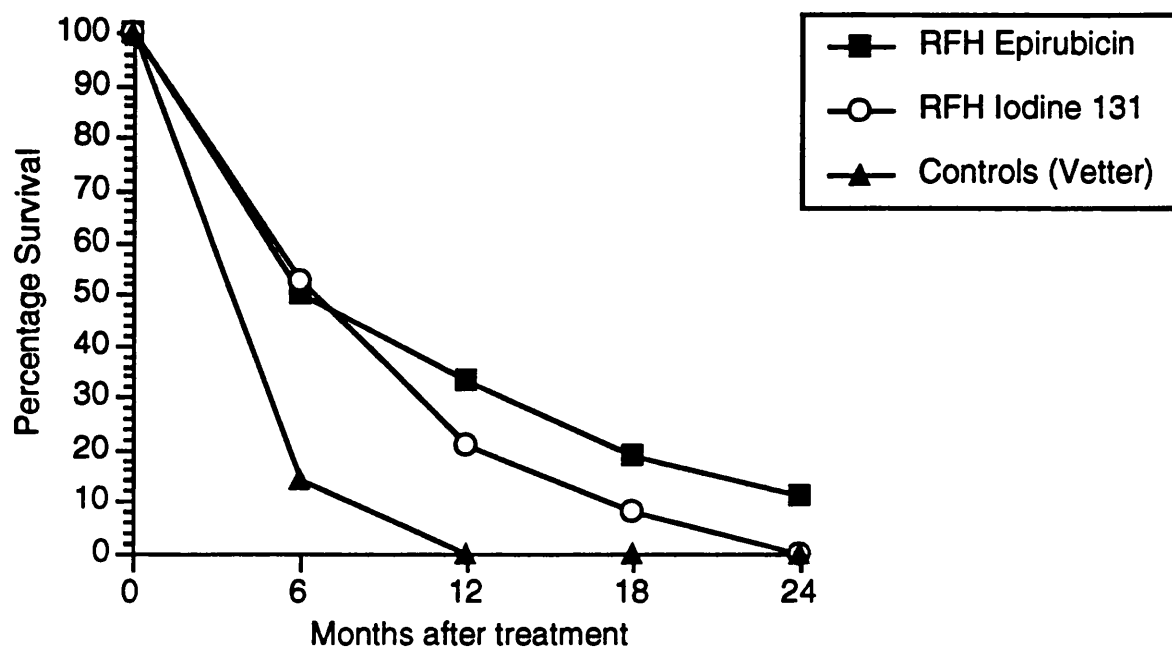
Unresectable Okuda Stage II Hepatocellular Carcinoma
treated with Lipiodol Epirubicin (n=29) or Lipiodol Iodine 131 (n=18),
compared with untreated controls (n=14) reported by Vetter et al.

Chapter 6

PROSPECTS FOR FURTHER EXPERIMENTAL RESEARCH

6.1   OUTLINE FOR FURTHER CELL CULTURE EXPERIMENTS

Cell lines other than Hep G2 and HUVECs

> *An anaplastic liver tumour cell line*
>
> *Cell lines of tumours arising in other organs (colon, breast)*
>
> *Tumour endothelium from HCCs*
>
> *Hepatocytes*
>
> *Phagocytes and fat storage cells*

Lipids other than Lipiodol

> *Individual fatty acids*
>
> *Other compound lipids*
>
> *The role of iodine*
>
> *The role of diatrizoates*

6.2   BEYOND CELL CULTURE STUDIES

Overcoming the limitations of cell culture

> *Use of three-dimensional matrices for cell culture*
>
> *Animal models*

Effects of malignant transformation on the cell membrane

Physical effect of lipids on tumour blood flow

6.3   IMPROVED DRUG DELIVERY

Liposomes

Lipophilic drug formulations

Modulation of drug uptake in tumour cells by Lipiodol or its component
> fatty acids

6.1 OUTLINE FOR FURTHER CELL CULTURE EXPERIMENTS

We now have an understanding of how tumour (HCC) cells and endothelial cells incorporate Lipiodol, and what effect that has on cellular metabolism and survival. Further studies from here on may proceed in several directions

Cell lines other than Hep G2 and HUVECs

An anaplastic liver tumour cell line

As opposed to Hep G2, which is well-differentiated, studies on an anaplastic cell line may lead to a better understanding of what results may be expected from Lipiodol-based therapies when applied across the entire spectrum of tumour diferentiation.

Other tumour cell lines, such as colonic, breast and renal carcinoma

The liver is the commonest site for colon cancer metastases, and while Lipiodol-targeted therapy can easily be delivered to these lesions, the clinical results have not been very encouraging so far. The interaction of colon cancer cells with Lipiodol may cast some light on why this is so, and how the results may be improved. Other common tumours such as breast and renal carcinoma should be studied to assess if Lipiodol uptake is an exclusive feature of HCCs or a common characteristic of most cancer cells.

Tumour endothelium

Though HUVECs have been used as the endothelial model in this study, the optimal model would be tumour endothelium derived from a human HCC. Methods for culture of hepatocyte subpopulations from a block of liver tissue have now become fairly standardised (170, 193). These techniques may be applied with some modifications to a resected specimen of HCC, to obtain cultures of tumour endothelium. It has also been shown that undifferentiated endothelial cells, when grown on a biomatrix derived from a specific organ, begin to express some of the phenotypic markers characteristic of the endothelium in that organ (194,

195). The use of tumour-derived biomatrix in a similar fashion may lead to endothelium that possesses tumour-related characteristics.

Primary cultures of Hepatocytes

These may be derived from freshly resected specimens of human liver or from livers harvested but not used for transplantation. Studies involving primary cultures of normal hepatocytes may provide valuable information on the differences between malignant cells and normal cells in the way they handle Lipiodol.

Phagocytes and fat storage cells

Though Kupffer cells are often absent in HCCs, some tumours do contain phagocytic cells in their ultrastructure (159). Also, cells resembling fat-storage (Ito) cells have been demonstrated in the perisinusoidal stroma of HCCs (161). Their role in Lipiodol retention remains to be clarified.

Lipids other than Lipiodol

Unsaturated fatty acids

Unsaturated fatty acids such as Linoleic acid, are the major constituent of Lipiodol, and the interaction of these individual fatty acids with tumour cells may lead to an understanding of which (if any) is the "active" constitiuent of Lipiodol primarily responsible for its uptake by the cells.

Other compound lipids

Other lipids such as olive oil and tea seed oil, have also been shown to be selectively retained by liver tumours in an animal model (80). Further studies comparing these to Lipiodol may lead to the identification of a better oily vehicle than Lipiodol.

The role of Iodine

The role of the iodine component of Lipiodol has not been assessed, and it may be useful to compare the cellular uptake of Lipiodol (and individual fatty acids) before and after iodination. Most of the in vivo dosimetry studies on Lipiodol have actually measured uptake of the iodine rather than the lipid component, though there is evidence to suggest that the two moieties will separate and be metabolised in different ways (89). The nature of the chemical bonding between the iodine and the lipid, and its strength in vitro and in vivo requires further clarification.

The role of Diatrizoates

Though sodium and meglumine diatrizoate frequently form a part of the drug-Lipiodol complex that is administered at chemotherapy, their individual interactions with the tumour cells remain to be analysed.

9.2 BEYOND CELL CULTURE STUDIES

Overcoming the limitations of cell culture

Use of three-dimensional matrices for cell culture

In conventional cell culture conditions it is difficult to replicate the in vivo situation of intact tissues, especially with regard to cell adhesion, distribution of nutrients and exchange of metabolic products. Initial attempts to remedy this involved the use of culture media enriched with supplemental growth factors, culturing the cells on substrates such as collagen, and co-culturing cell populations e.g. hepatocytes and epithelial cells. The use of hepatocyte 'couplets' (cell clusters generated by limited collagenase digestion) and thin slices of liver tissue have been reported (193). Recently, there have been reports of three-dimensional culture systems using polyurethane discs wherein cells can survive as multicellular complexes (196), and perfusion apparatuses which attempt to replicate the organotypic environment (197). The primary aim behind the development of these models has been the creation of a bio-artificial liver to provide extra-corporeal support to patients with liver failure.

166

But using malignant cells, it may be possible to utilise these systems as *in vitro* models of a tumour.

Animal models

The VX2 carcinoma, implanted into the rabbit liver, has been the most commonly used animal model for liver cancer. However, it is an anaplastic tumour derived originally from a papilloma. Besides, there are difficulties in extrapolating findings from a rabbit tumour to the human situation. Implantation of human HCC cells into the livers of 'nude' mice may provide a more appropriate model, and has been attempted with some success (D. Shouval, personal communication). However, the procedure is technically difficult, and is yet to find widespread acceptance.

Effects of malignant transformation on the cell membrane

Malignant transformation (chemical carcinogeneseis) has been associated with biochemical changes in cell membranes, including alterations in lipid composition, fatty acid saturation, enzyme expression and receptor turnover (198). These changes may possibly lead to preferential uptake of certain lipids by the tumour cells, but their role - if any - in Lipiodol retention is as yet unclear.

Physical effect of lipids on tumour blood flow

The passage of arterially injected Lipiodol through the parenchyma of the porcine liver has been mapped by Kan, Ivancev and co-workers in a series of elegant experiments involving *in vivo* microscopy (199). It may be possible to extend this technique to liver tumours in an animal model. Retention of Lipiodol may be related to alterations in blood viscosity due to injection of the lipid, and consequent changes in hepatic arterial and tumour blood flow; but there is little experimental data to support this hypothesis. It may be possible to assess the effect of Lipiodol on blood viscosity by thromboelastography. This is a technique that measures the visco-elastic properties of clotting blood, displaying a visual trace of all phases of coagulation and fibrinolysis (200).

6.3 IMPROVED DRUG DELIVERY

Liposomes

There is evidence to suggest that the size of the oil droplets in a drug-Lipiodol emulsion plays an important role in its biodistribution (91), and may determine the extent of Lipiodol uptake by different cell populations within the liver (157). Droplet size in an emulsion may be standardised by use of an ultrasonic agitator. Cell culture experiments using standardised droplet sizes may yield further information on how droplet size might be manipulated to improve drug delivery.

Liposomes consist of one or more concentric layers of phospholipid, with an aqueous phase trapped between the lipid layers and in the core. They may therefore be used to deliver lipid-soluble and water-soluble drugs to target tissues. The use of liposomal carrier systems in targeting cytotoxic drugs to tumours in an animal model has been associated with decrease in tumour size and prolonged survival (201). Liposomes with Lipiodol incorporated into the lipid phase, and a cytotoxic agent in the aqueous phase may have some advantages compared to other delivery systems.

Lipophilic drug formulations

The use of lipophilic formulations with Lipiodol (as opposed to the majority of currently available formulations, which are lipophobic) is likely to enhance drug uptake, and warrants evaluation. The successful clinical use of SMANCS (a lipophilic macromolecular compound including styrene, maleic acid and neocarcinostatin) has been reported from Japan, but this drug has not been compared with other anticancer agents within the framework of a prospective clinical trial, and its usage has yet to find widespread acceptance. A lipophilic ester of 5-Fluorouracil, FUdR, has been used in clinical studies, and compared favourably against Doxorubicin in a randomised trial (117). Of the anthracyclines, the recently synthesised Idarubicin is reported to be more lipophilic compared to doxorubicin and epirubicin.

Modulation of cytotoxic drug uptake in tumour cells by Lipiodol

Pre-treatment by essential fatty acids such as gamma-linoleic acid and eicosapentanoic acid has been shown to enhance the accumulation of cytotoxic agents within tumour cells in culture (202). Lipiodol is composed of fatty acid esters and may possess similar properties. The cell culture system developed in this study could be utilised for such an investigation, using flow cytometry to assess cellular levels of intrinsically fluorescent anthracyclines.

References

1.   Kojiro M, Nakashima T. Pathology of hepatocellular carcinoma. In: Okuda K, Ishak KG, ed. Neoplasms of the liver. Tokyo: Springer-Verlag, 1987: 81-84.

2.   Sherlock S, Dooley J. Diseases of the liver and biliary system. (9th ed.) Oxford: Blackwell Scientific Publications, 1993

3.   Lin TY. Malignant tumours of the liver. In: Bockus, ed. Gastroenterology. 3rd ed. Philadelphia: WB Saunders, 1976: 522-534.

4.   Hodgson HJF. Primary hepatocellular carcinoma. In: Blumgart LH, ed. Surgery of the liver and biliary tract. Edinburgh: Churchill Livingstone, 1988: 1129-1133.

5.   Higginson J. The geographical pathology of primary liver cancer. Cancer Research 1963;23:1624-28.

6.   Wong J, Choi TK. Primary liver cell cancer - Asian experience. In: Blumgart LH, ed. Surgery of the liver and biliary tract. Edinburgh: Churchill Livingstone, 1988: 1135-1151.

7.   Berman C. Primary carcinoma of the liver. London: H. K. Lewis, 1951

8.   MacSween RNM, Scott AR. Hepatic cirrhosis: A clinicopathological review of 520 cases. Journal of Clinical Pathology 1973;26:936-945.

9.   Johnson PJ, Krasner N, Portmann B, Eddleston ALWF, Williams R. Hepatocellular carcinoma in Great Britain: Influence of age, sex, HBsAg status and aetiology of underlying cirrhosis. Gut 1978;19:1022-1033.

10.   Anthony PB. Liver cell dysplasia: What is its significance? Hepatology 1987;7:394.

11. Beasley RP, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and Hepatitis B virus: A prospective study of 22707 men in Taiwan. Lancet 1981;2(8256):1129-1133.

12. Bassendine MF, Seta L, Salmeron J, et al. Incidence of hepatitis B virus infection in alcoholic liver disease, HBsAg negative chronic active liver disease and primary liver cell cancer in Britain. Liver 1983;3(2):65-70.

13. Shafritz DA, Shouval D, Sherman HI, Hadziyannis SJ, Kew MC. Integration of hepatitis B virus DNA into the genome of liver cells in chronic liver disease and hepatocellular carcinoma. New England Journal of Medicine 1981;305:1067-1073.

14. Popper H, Gerber MA, Thung SN. The relation of hepatocellular carcinoma to infection with hepatitis B and related viruses in man and animals. Hepatology 1982;2:1S.

15. Nishioka K, watanabe J, Furuta S, et al. A high prevalence of antibody to the hepatitis C virus in patients with hepatocellular carcinoma in Japan. Cancer 1991;67(2):429-433.

16. Colombo M, Franchis RD, Ninno ED, et al. Hepatocellular carcinoma in Italian patients with cirrhosis. New England Journal of Medicine 1991;325:675-680.

17. Bruix J, Barrera JM, Calvet X, et al. Prevalence of antibodies to hepatitis C virus in Spanish patients with hepatocellular carcinoma and hepatic cirrhosis. Lancet 1989;2(8670):1004-1006.

18. Kew MC, Houghton M, Choo Q-L, et al. Hepatitis C virus antibodies in Southern African blacks with hepatocellular carcinoma. Lancet 1990;335(8694):873-874.

19. Hardell L, Bengtsson NO, Jonsson U, et al. Aetiological aspects of primary liver cancer with special regard to alcohol, organic solvents and acute intermittent porphyria - an epidemiological investigation. British Journal of Cancer 1984;50(3):389-397.

20.   Bosch FX, Peers F. Relevance to human cancer of N-nitroso compounds, tobaco smoke and mycotoxins. In: Bartsch H, ed. IARC Scientific Publications No. 105. Lyons: International Agency for Research on Cancer, 1991.

21.   Ozturk M. p53 mutation in hepatocellular carcinoma after aflatoxin exposure. Lancet 1991;338(8779):1356-1359.

22.   Okuda K, Peters RL, Simson I. Gross anatomical features of hepatocellular carcinoma from three disparate geographic areas: Proposal of a new classification. Cancer 1984;54:2165-2173.

23.   Ferrell LD, Crawford JM, Dhillon AP, Scheuer PJ, Nakanuma Y. Proposal for standardized criteria for the diagnosis of benign, borderline and malignant hepatocellular lesions arising in chronic advanced liver disease. American Journal of Surgical Pathology 1993;17(11):1113-1123.

24.   Dusheiko GM, Hobbs KE, Dick R, Burroughs AK. Treatment of small Hepatocellular Carcinomas. Lancet 1992;340(8814):285-288.

25.   Tang ZY, Yu YR, Zhou XD, Yang BH, Ma ZC, Lin ZY. Subclinical Hepatocellular Carcinoma: An analysis of 391 patients. Journal of Surgical Oncology 1993;(Supplement 3):55-58.

26.   Nomura F, Ohnishi K, Tanabe Y. Clinical features and prognosis of Hepatocellular Carcinoma with reference to serum Alpha fetoprotein levels. Cancer 1989;64(8):1700-1707.

27.   Nakao A, Virji A, Iwaki Y, Carr B, Iwatsuki S, Starzl T. Abnormal Prothrombin (Des-gamma-carboxy-prothrombin) in Hepatocellular Carcinoma. Hepatogastroenterology 1991;38(5):316-323.

28.   Bukofzer S, Stass PM, Kew MC, deBeer M, Groeneveld HT. Alpha-L-fucosidase as a serum marker for Hepatocellular Carcinoma in South African blacks. British Journal of Cancer 1989;59(3):417-420.

29.   Okuda K, Ohtsuki T, Obata H, et al. Natural history of

hepatocellular carcinoma and prognosis in relation to treatment. Cancer 1985;56:918-928.

30.   Hermanek P, Sobin LH, ed. TNM Classification of Malignant Tumours. 4th ed. Berlin: Springer-Verlag, 1987:53-55.

31.   Lee NW, Wong J, Ong GB. The surgical management of primary cancer of the liver. World Journal of Surgery 1982;6:66-75.

32.   Vetter D, Wenger J-J, Bergier J-M, Doffoel M, Bockel R. Transcatheter oily chemoembolisation in the management of advanced hepatocellular carcinoma in cirrhosis: Results of a western comparative study in 60 patients. Hepatology 1991;13:427-433.

33.   Okuda K. Primary liver cancers in Japan. Cancer 1980;45:2663-2675.

34.   Anonymous. Primary liver cancer in Japan. Clinicopathologic features and results of surgical treatment. Liver cancer study group of Japan. Annals of Surgery 1990;211(3):277-287.

35.   Adson MA. Primary cancer of the liver: considerations about resection. In: Bengmark S, ed. Progress in surgery of the liver, pancreas and biliary system. Dordrecht, Holland: Martinus Nijhoff, 1988: 179-188.

36.   Maraj R, Kew MC, Hyslop RJ. Resectability rate of Hepatocellular carcinoma in rural South Africans. British Journal of Surgery 1988;75:335-338.

37.   Fortner JG, Maclean BJ, Kim DK, et al. The seventies evolution in surgery for liver cancer. Cancer 1981;47:2162-2166.

38.   Adson MA. Primary hepatocellular cancers - Western experience. In: Blumgart LH, ed. Surgery of the Liver and Biliary Tract. Edinburgh: Churchill Livingstone, 1988: 1153-1165.

39.   Belghiti J. Resection of Hepatocellular carcinoma complicating cirrhosis. British Journal of Surgery 1991;78:257-258.

40. Lin TY. Resectional therapy for primary malignant hepatic tumours. In: Murphy GP, ed. International advances in surgical oncology. New York: Alan R. Liss, 1979: 25-54. vol 2.

41. Penn I. Hepatic transplantation for primary and metastatic cancers of the liver. Surgery 1991;110:726-735.

42. Iwatsuki S, Starzl TE, Sheahan DG, et al. Hepatic Resection versus Transplantation for Hepatocellular Carcinoma. Annals of Surgery 1991;214(3):221-228.

43. Bismuth H, Chiche L, Adam R, Castaing D, Diamond T, Dennison A. Liver resection versus transplantation for Hepatocellular carcinoma in cirrhotic patients. Annals of Surgery 1993;218(2):145-151.

44. Patt YZ, Claghorn L, Charnsangavej C, Soski M, Cleary K, Mavligit GM. Hepatocellular carcinoma: A retrospective analysis of treatments to manage disease confined to the liver. Cancer 1988;61:1884-1888.

45. Falkson G, Coetzer B. Chemotherapy of primary liver cancer. In: Okuda K, Ishak KG, ed. Neoplasms of the liver. Tokyo: Springer-Verlag, 1987: 321-326.

46. Dunk AA, Scott SC, Johnson PJ, et al. Mitozantrone as single agent therapy in hepatocellular carcinoma - a phase II study. Journal of Hepatology 1985;1:395-404.

47. Cochrane AMG, Murray-Lyon IM, Brinkley DM, et al. Quadruple therapy versus radiotherapy in the treatment of primary hepatocellular carcinoma. Cancer 1977;40:609-614.

48. Houle S, Yip T, Shepherd F, et al. Hepatocellular carcinoma: Pilot trial of treatment with Y-90 microspheres. Radiology 1989;172:857-860.

49. Ohto M, Ebara M, Yoshikama M, Okuda K. Radiation therapy and percutaneous ethanol injection for the treatment of hepatocellular carcinoma. In: Okuda K, Ishak KG, ed. Neoplasms of the liver. Tokyo:

Springer-Verlag, 1987: 335-341

50.   Livraghi T, Salmi A, Bolondi L, et al. Small hepatocellular carcinoma: percutaneous alcohol injection - results in 23 patients. Radiology 1988;168:313-317.

51.   Shiina S, Tagawa K, Niwa Y, et al. Percutaneous ethanol injection therapy for hepatocellular carcinoma: Results in 146 patients. American Journal of Roentgenology 1993;160(5):1023-1028.

52.   Livraghi T, Bolondi L, Lazzaroni S, et al. Percutaneous ethanol injection in the treatment of hepatocellular carcinoma in cirrhosis. A study on 207 patients. Cancer 1992;69(4):925-929.

53.   Gilbert JC, Onik GM, Hoddick WK, Rubinski B. Real time ultrasonic monitoring of hepatic cryosurgery. Cryobiology 1985;22:319-330.

54.   Ravikumar TS, Kane R, Cady B, Jenkins R, Clouse M, Steele G. A 5-year study of cryosurgery in the treatment of liver tumours. Archives of Surgery 1991;126:1520-1524.

55.   Dowlatshahi K, Bhattacharya AK, Silver B, Matalon T, Williams JW. Percutaneous interstitial laser therapy of a patient with recurrent hepatoma in a transplanted liver. Surgery 1991;112:603-606.

56.   Nakhgevany KB, Mobini J, Bassett JG, Miller E. Nonabsorbable radioactive material in the treatment of carcinoma by local injections. Cancer 1988;61:931-940.

57.   Meyers WC, Jones RS. Internal radiation for bile duct cancer. World Journal of Surgery 1988;12:99-104.

58.   Markham N, Ritson A, James O, et al. Primary hepatocellular carcinoma localised by a radiolabelled monoclonal antibody. Journal of Hepatology 1986;2:25-31.

59.   Order SE, Stillwagon GB, Klein JL, et al. Iodine 131 antiferritin, a

new treatment modality in hepatoma: a radiation therapy oncology group study. Journal of Clinical Oncology 1985;3:1573-1582.

60.   Sitzmann JV, Order SE, Klein JL, et al. Conversion by new treatment modalities of nonresectable to resectable hepatocellular cancer. Journal of Clinical Oncology 1987;5:1566-1573.

61.   Order SE, Vriesendorp HM, Klein JL, Leitchner PK. A phase I study of 90-Yttrium antiferritin: dose escalation and tumour dose. Antibody, Immunoconjugates and Radiopharmaceuticals 1988;1:163-168.

62.   Zhang XF, Klein JL, Order SE. Quantitative comparison of tumour dose of radiolabelled monoclonal and polyclonal antibodies in an experimental tumour model. Antibody, Immunoconjugates and Radiopharmaceuticals 1988;1:35.

63.   Lin D-Y, Liaw Y-F, Lee T-Y, Lai C-H. Hepatic arterial embolisation in patients with unresectable hepatocellular carcinoma - a randomised controlled trial. Gastroenterology 1988;94:453-456.

64.   Breedis C, Young G. The blood supply of neoplasms of the liver. American Journal of Pathology 1954;30:969-974.

65.   Yamada R, Kishi K, Tsuda M, Kawabata M, Sato M. Embolotherapy for the unresectable hepatoma. Gann Monograph on Cancer Research 1991;38:131-147.

66.   Nakao N, Kamino K, Miura K, et al. Recurrent hepatocellular carcinoma after partial hepatectomy: value of treatment with transcatheter arterial chemoembolisation. American Journal of Roentgenology 1991;156:1177-1179.

67.   Hemingway AP, Allison DJ. Complications of embolisation: analysis of 410 procedures. Radiology 1988;166:669-672.

68.   Jeng K-S, Ching H-J. The role of surgery in the management of unusual complications of transcatheter arterial embolisation for hepatocellular carcinoma. World Journal of Surgery 1988;12:362-368.

69.   Hwang T-L, Chen M-F, Lee T-Y, Chen T-J, Lin D-Y, Liaw Y-F. Resection of hepatoellular carcinoma after transcatheter arterial embolisation. Archives of Surgery 1987;122:756-759.

70.   Nagasue N, Galizia G, Kohno H, et al. Adverse effects of preoperative hepatic artery chemoembolisation for resectable hepatocellular carcinoma: A retrospective comparison of 138 liver resections. Surgery 1989;106:81-86.

71.   Sasaki Y, Imaoka S, Kasugai H, et al. A new approach to chemoembolisation therapy for hepatoma using ethiodised oil, Cisplatin and Gelatin sponge. Cancer 1987;60:1194-1203.

72.   Sicard JA, Forestier G. Injections intravasculaires d'huile iodee sous control radiologique. Comptes rendus des seances de la Societe de Biologie et des ses filiales 1923;88:1200.

73.   Reynolds JEF, ed. Martindale: The Extra Pharmacopoeia. 2nd ed. London: The Pharmaceutical Press, 1989:866.

74.   Idezuki Y, Sugiura M, Hatano S, Kimoto S. Hepatography for detection of small mass in liver: experience with oily contrast medium. Surgery 1966;60:566-572.

75.   Nakakuma K, Tashiro S, Uemura K, et al. Studies on anticancer treatment with an oily anticancer drug injected into the ligated hepatic artery for hepatic cancer. Nichidoku Iho 1979;24:675-682.

76.   Konno T. Targeting chemotherapy: experimental and clinical study of arterial administration of SMANCS/Lipiodol.Tokyo: Yubunsha, 1987

77.   Okayasu I, Hatakeyama S, Yoshida T, et al. Selective and persistent deposition and gradual drainage of iodised oil in hepatocellular carcinoma after injection into the feeding hepatic artery. American Journal of Clinical Pathology 1988;90:536-544.

78.   Ohishi H, Uchida H, Yoshimura H, et al. Hepatocellular carcinoma detected by iodised oil. Radiology 1985;154:25-29.

79.   Chastin I. Composition of Lipiodol Ultra Fluide. Paris: Laboratoire Guerbet, 1992.

80.   Iwai K, Maeda H, Konno T. Tumour targeting by arterial administration of lipids: rabbit model with VX2 carcinoma in the liver. Anticancer Research 1987;7:321-328.

81.   Kanematsu T, Inokuchi K, Sugimachi K, et al. Selective effects of lipiodolised anti-tumour agents. Journal of Surgical Oncology 1984;25:218-226.

82.   Novell JR. The action of Lipiodol on liver tumours [Master of Surgery]. University of Cambridge, 1992.

83.   Nakamura H, Hashimoto T, Oi H, Sawada S. Transcatheter oily chemoembolisation of hepatocellular carcinoma. Radiology 1989;170(3):783-786.

84.   Raoul JL, Heresbach D, Bretagne JF, et al. Chemoembolization of hepatocellular carcinomas: A study of the biodistribution and pharmacokinetics of doxorubicin. Cancer 1992;70(3):585-590.

85.   Raoul J-L, Bourguet P, Bretagne J-F, et al. Hepatic artery injection of I-131-labelled lipiodol. Part I: Biodistribution study results in patients with hepatocellular carcinoma and liver metastases. Radiology 1988;168:541-545.

86.   Madsen MT, Park CH, Thakur ML. Dosimetry·of Iodine 131 Ethiodol in the treatment of hepatoma. Journal of Nuclear Medicine 1988;29:1038-1044.

87.   Nakajo M, Kobayashi H, Shimabukwo K, et al. Biodistribution and in vivo kinetics of Iodine 131 lipiodol infused via the hepatic artery of patients with hepatic cancer. Journal of Nuclear Medicine 1988;29:1066-1077.

88. Iwai K, Maeda H, Konno T. Use of oily contrast medium for selective drug targeting to tumour: enhanced therapeutic effect and x-ray image. Cancer Research 1984;44:2115-2121.

89. Dalion J, Guerbet M, Delaville A. Destinée du lipiodol injecté par voie intraveineuse ou lymphatique: modifications histologiques consécutives à ces injections. Therapie 1965;20:789-796.

90. Hind RE, Loizidou M, Perring S, et al. Biodistribution of lipiodol following hepatic arterial injection. British Journal of Surgery 1992;79:952-954.

91. Hellekant C, Olin T. Effects of emulsified iodo-lipids: A toxicologic investigation in the rabbit. Acta Radiologica Diagnosis 1976;17:223-234.

92. Kusumoto S, Imamura A, Watanabe K. Case report: Incidental lipid embolisation to the brain and kidney after lymphography in a patient with malignant lymphoma. Clinical Radiology 1991;44:279-280.

93. Hirota S, Sako M, Fujita Y, Nagae T, Sugimoto K, Kono M. Hemodynamic changes of the liver after hepatic arterial embolisation. Proceedings of the Joint meeting of the Cardiovascular and Interventional Radiologic Society of Europe and the Society of Cardiovascular and Interventional Radiology (USA). 1991: 20.

94. Bismuth H, Morino M, Sherlock D, et al. Primary treatment of hepatocellular carcinoma by arterial chemoembolisation. American Journal of Surgery 1992;163:387-394.

95. Beppu T, Ohara C, Yamaguchi Y, et al. A new approach to chemoembolisation for unresectable hepatocellular carcinoma using aclarubicin microspheres in combination with cisplatin suspended in iodised oil. Cancer 1991;68:2555-2560.

96. Civalleri D, Scopinaro G, Balletto N, et al. Changes in vascularity of liver tumours after hepatic arterial embolisation with degradable

179

starch microspheres. British Journal of Surgery 1988;76:699-703.

97.   Demachi H, Matsui O, Miwa A, et al. Changes in intrahepatic microvasculature after hepatic arterial embolisation in patients with hepatocellular carcinoma. 4th International Symposium of Interventional Radiology and New Vascular Imaging.   Kumamoto, Japan: , 1992: 155.

98.   Ohkusa A. Intrahepatic microvacular changes after intrahepatic arterial injection of of lipiodol in normal and cirrhotic rats. Nippon Igaku Hoshasen Gakkai Zashi 1992;52(6):774-785.

99.   Konno T. Targeting chemotherapy for hepatoma: Arterial administration of anticancer drugs dissolved in lipiodol. European Journal of Cancer 1992;28:403-409.

100.   Yumoto Y, Jinno K, Tokuyama K, et al. Hepatocellular carcinoma detected by iodised oil. Radiology 1985;154:19-24.

101.   Hayashi N, Yamamoto K, Tamaki N, et al. Metastatic nodules of hepatocellular carcinoma: Detection with angiography, CT and ultrasonography. Radiology 1987;165:61-63.

102.   Choi BI, Park JH, Kim BH, Kim SH, Han MC, Kim C-W. Small hepatocellular carcinoma: Detection with sonography, computed tomography (CT), angiography and lipiodol CT. British Journal of Radiology 1989;62:897-903.

103.   Kondo S, Nishikawa M, Takami S, et al. Diagnostic value of lipiodol injection in focal nodular hyperplasia of the liver. American Journal of Gastroenterology 1991;86(6):779-781.

104.   Choi BI, Kim HC, Han JK. Therapeutic effect of transcatheter oily chemoembolisation for encapsulated nodular hepatocellular carcinoma: CT and pathologic findings. Radiology 1992;182:709-713.

105.   Maki S, Konno T, Maeda H. Image enhancement in computed tomography for sensitive diagnosis of liver cancer and semi-

quantitation of tumour selective drug targeting with oily contrast media. Cancer 1985;56:751-757.

106. Matsui O, Takashima T, Kadoya M, et al. Liver metastases from colorectal cancers: Detection with CT during arterial portography. Radiology 1987;165:65-69.

107. Lewis E, AufderHeide JF, Bernardino ME, Barnes PA, Thomas JL. CT detection of hepatic metastases with Ethiodised Oil Emulsion 13. Journal of Computer Assisted Tomography 1982;6(6):1108-1114.

108. Burgener FA, Violante MR. Comparison of hepatic VX2 carcinoma after intra-arterial, intra-portal and intra-parenchymal tumour cell injection. Investigative Radiology 1979;14(5):410-414.

109. Fukushima S, Kawaguchi T, Nishida M, et al. Selective anticancer effects of 3',5'-Dioctanoyl-5-fluoro-2'-deoxyuridine, a lipophilic prodrug of 5-Fluoro-2'-deoxyuridine, dissolved in an oily lymphographic agent on hepatic cancer of rabbits bearing VX2 tumour. Cancer Research 1987;47:1930-34.

110. Furuta T, Kanematsu T, Ezaki T, Sugimachi K, Inokuchi K. Anti-tumour effect of lipiodolised doxorubicin on VX2 carcinoma in rabbits. Japanese Journal of Surgery 1985;15(1):89-91.

111. Konno T. Targeting cancer chemotherapeutic agents by use of lipiodol conrast medium. Cancer 1990;66(9):1897-1903.

112. Tsai C, Kusumoto Y, Harada R, et al. Effect of intrahepatic arterial infusion of 131-I-labelled lipiodol on hepatocellular carcinoma of the rat. Annals of the Academy of Medicine 1986;15(4):521-524.

113. Takayasu K, Shima Y, Muramatsu Y, et al. Hepatocellular carcinoma: treatment with intra-arterial iodised oil with and without chemotherapeutic agents. Radiology 1987;162:345-351.

114. Kanematsu T, Furuta T, Takenaka K, et al. A 5-year experience of lipiodolisation: selective regional chemotherapy for 200 patients with

hepatocellular carcinoma. Hepatology 1989;10:98-102.

115. Tanaka K, Okazaki H, Nakamura S, et al. Hepatocellular carcinoma: Treatment with a combination therapy of transcatheter arterial embolisation and percutaneous ethanol injection. Radiology 1991;179:713-717.

116. Young N, Hollands M, Wong KP. Symptom relief and survival after chemo-embolisation with adriamycin, lipiodol and gelfoam for hepatocellular carcinoma. Australasian Radiology 1993;37(2):173-176.

117. Yamashita Y, Takahashi M, Shinzato J, Bussaka H, Baba Y, Oguni T. Evaluation of oil-soluble FUdR ester for transcatheter arterial treatment of hepatomas. Annals of Oncology 1992;3:367-370.

118. Kirk S, Blumgart R, Craig B, Rosen A, Terblanche J, Spence RAJ. Irresectable hepatoma treated by intrahepatic iodised oil doxorubicin hydrochloride: Initial results. Surgery 1991;109:694-697.

119. Leung WT, Shiu WCT, Leung N, et al. Treatment of inoperable hepatocellular carcinoma by intra-arterial lipiodol and 4-epidoxorubicin. Cancer Chemotherapy and Pharmacology 1992;29:401-404.

120. Novell JR, Dusheiko G, Markham NI, Reddy K, Dick R, Hobbs KEF. Selective regional chemotherapy of unresectable hepatic tumours using Lipiodol. HPB Surgery 1991;4:223-236.

121. Shibata J, Fujiyama S, Sato T, Kishimoto S, Fukushima S, Nakano M. Hepatic arterial injection chemotherapy with cisplatin suspended in an oily lymphographic agent for hepatocellular carcinoma. Cancer 1989;64:1586-1594.

122. Burton MA, Gray BN, Klemp PF, Kelleher DK, Hardy N. Selective internal radiation therapy: distribution of radiation in the liver. European Journal of Cancer and Clinical Oncology 1989;25:1487-1491.

123. Herba MJ, Illescas FF, Thirlwell MP, et al. Hepatic malignancies: improved treatment with intra-arterial Y-90. Radiology 1988;169:311-314.

124. Ehrhardt GJ, Day DE. Therapeutic use of Y-90 microspheres. Journal of Nuclear Biology and Medicine 1987;14:233-242.

125. Blanchard RJ, Morrow IM, Sutherland JB. Treatment of liver tumours with Yttrium-90 microspheres alone. Canadian Association of Radiologists Journal 1989;40:206-210.

126. Wollner I, Knutsen C, Smith P, et al. Effects of hepatic arterial Yttrium 90 glass microspheres in dogs. Cancer 1988;61:1336-1344.

127. Meade VM, Burton MA, Gray BN, Self GW. Distribution of different sized microspheres in experimental hepatic tumours. European Journal of Cancer and Clinical Oncology 1987;23:37-41.

128. Bretagne J-F, Raoul J-L, Bourguet P, et al. Hepatic artery injecton of I-131-labeled lipiodol. PartII. Preliminary results of therapeutic use in patients with hepatocellular carcinoma and liver metastases. Radiology 1988;168:547-550.

129. Kobayashi H, Hidaka H, Kajiya Y, et al. Treatment of hepatocellular carcinoma by transarterial injection of anticancer agents in iodised oil suspension or of radioactive iodised oil solution. Acta Radiologica (Diagn) 1986;27:139-147.

130. Park CH, Suh JH, Yoo HS, Lee JT, Kim DI, Kim BS. Treatment of hepatocellular carcinoma with radiolabelled lipiodol: a preliminary report. Nuclear Medicine Communications 1987;8:1075-1087.

131. Raoul J-L, Bretagne J-F, Caucanas JP. Internal radiation therapy for hepatocellular carcinoma: results of a French multicenter phase II trial of transarterial injection of Iodine 131-labeled lipiodol. Cancer 1992;69:346-352.

132. Yoo HS, Lee JT, Kim KW, et al. Nodular hepatocelular carcinoma: Treatment with subsegmental intra-arterial injection of Iodine 131-labeled iodised oil. Cancer 1991;68:1878-1884.

133.   Yu Y-Q, Xu D-B, Zhou X-D, Lu J-Z, Tang Z-Y, Mack P. Experience with liver resection after hepatic arterial chemoembolisation for hepatocellular carcinoma. Cancer 1993;71(1):62-65.

134.   Allison DJ. Embolisation of liver tumours. In: Blumgart LH, ed. Surgery of the Liver and Biliary Tract. Edinburgh: Churchill Livingstone, 1988: 1201-1217.

135.   Adachi E, Matsumata T, Nishizaki T, Hashimoto H, Tsuneyoshi M, Sugimachi K. Effects of preoperative transcatheter hepatic arterial chemoembolisation for hepatocellular carcinoma. Cancer 1993;72(12):3593-3598.

136.   Nagasue N, Kohno H, Uchida M. Evaluation of preoperative transcatheter arterial embolisation in the treatment of resectable liver cancer. Seminars in Surgical Oncology 1993;9(4):327-331.

137.   Morino M, Miglietta C, Grosso M, Giuli MD, Bismuth H. Preoperative chemoembolisation for hepatocellular carcinoma. Journal of Surgical Oncology - Supplement 1993;3:91-93.

138.   Izumi R, Shimizu K, Iyobe T, et al. Postoperative adjuvant hepatic arterial infusion of Lipiodol containing anticancer drugs in patients with hepatocellular carcinoma. Hepatology 1994;20:295-301.

139.   Spence RAJ, Rosen A, Krige JEJ, Blumgart RL, Temple-Camp CRE, Terblanche J. Unresectable fibrolamellar hepatocellular carcinoma treated with intra-arterial lipiodolised doxorubicin. South African Medical Journal 1987;72:701-703.

140.   Ogita S, Tokiwa K, Taniguchi H, Takahashi T. Intraarterial chemotherapy with lipid contrast medium for hepatic malignancies in infants. Cancer 1987;60:2886-2890.

141.   Park CH, Suh JH, Yoo HS, Lee JT, Kim DI, Lee KS. Iodine 131 labelled lipiodol retention within hepatic cavernous haemangioma. Radiology 1987;163:283-284.

142. Yamamoto T, Kawarada Y, Yano T, Noguchi T, Mizumoto R. Spontaneous rupture of haemangioma of the liver: Treatment with transcatheter hepatic arterial embolisation. American Journal of Gastroenterology 1991;86(11):1645-1649.

143. Kobayashi H, Inoue H, Shimada J, et al. Intra-arterial injection of Adriamycin/Mitomycin C lipiodol suspension in liver metastases. Acta Radiologica 1987;28:275-280.

144. Konno T, Maeda H, Iwai K, et al. Selective targeting of anti-ancer drug and simultaneous image enhancemernt in solid tumours by arterially administered lipid contrast medium. Cancer 1984 1984;54:2367-2374.

145. Kobayashi M, Maeda H, Imai K, Konno T, Sugihara S, Yamanaka H. Tumor-targeted chemotherapy with lipid contrast medium and macromolecular anticancer drug (SMANCS) for renal cell carcinoma. Urology 1991;37(3):288-294.

146. Novell JR, Parbhoo SP, Dawson K, Dick R, Kelleher SM. Targeted treatment for recurrent breast carcinoma with regional lipiodol/epirubicin infusion. Lancet 1990;336:1383.

147. Ozono S, Okajima E, Hirao Y, et al. Transcatheter arterial embolisation of vesical artery in the treatment of invasive bladder carcinoma. European Urology 1988;15:176-179.

148. Miller DL, O'Leary TJ, Girton M. Distribution of iodized oil within the liver after hepatic arterial injection. Radiology 1987;162:849-852.

149. Arnold MM, Kreel L, Wallace AC, Li AKC. Distribution of lipiodol and evidence for tumor necrosis in hepatocellular carcinoma. American Journal of Clinical Pathology 1992;97:405-410.

150. Kan Z, Ivancev K, Hagerstrand I, Chuang VP, Lunderquist A. In vivo microscopy of the liver after injection of lipiodol into the hepatic artery and portal vein in the rat. Acta Radiologica 1989;30:419-425.

151. Nakashima T. Vascular changes and haemodynamics in hepatocellular carcinoma. In: Okuda K, Peters RL, ed. Hepatocellular Carcinoma. New York: John Wiley, 1976: 169-203.

152. Peterson HI, Appelgren KL. Experimental studies on the uptake and retention of labeled proteins in a rat tumour. European Journal of Cancer 1973;9:543-547.

153. Shibita K, Okudo H, Ishibashi H, et al. Rat alpha1-acid glycoprotein: uptake by inflammatory and tumour tissue. British Journal of Experimental Pathology 1978;59:601-608.

154. Senger DR, Galli SJ, Dvorak AM, et al. Tumour cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983;291:983-985.

155. Abrams HL. The response of neoplastic renal vessels to epinephrine in man. Radiology 1964;82:217-224.

156. Suzuki M, Hori K, Abe I, et al. A new approach to cancer chemotherapy: selective enhancement of tumour blood flow with angiotensin II. Journal of the National Cancer Institute 1981;65:663-669.

157. Ivancev K, Lunderquist A, McCuskey R, McCuskey P, Wretlind A. Effect of intravenously injected iodinated lipid emulsions on the liver. Acta Radiologica 1989;30:291-298.

158. Ivancev K, Lunderquist A, McCluskey R, McCluskey P, Wretlind A. Experimental investigation of a new iodinated lipid emulsion for computed tomography of the liver. Acta Radiologica 1989;30:1-7.

159. Ichida T, Hata K, Yamada S, et al. Subcellular abnormalities of liver sinusoidal lesions in human hepatocellular carcinoma. Journal of Submicroscopic Cytology and Pathology 1990;22(2):221-229.

160. Vermess M, Lau D, Adams MD, et al. Biodistribution studies of ethiodised oil emulsion 13 for computed tomography of the liver and

spleen. Journal of Computer Assisted Tomography 1982;6:1115-1119.

161. Enzan H, Himeno H, Iwamura S, et al. Alpha-smooth muscle actin-positive perisinusoidal stromal cells in human hepatocellular carcinoma. Hepatology 1994;19:895-903.

162. Wood CB, Habib NA, Thompson NA, et al. Increase of oleic acid in erythrocytes associated with malignancy. British Medical Journal 1985;291:163-165.

163. Habib NA, Wood CB, Apostolov K, Kirsten D, Smadja C, Coutinho J. Reduction in the stearic to oleic acid ratio in the circulating red blood cells: a possible tumour marker in solid human neoplasms. Cancer Detection and Prevention 1984;7:477.

164. Apostolov K, Barker W, Catorsky D, et al. Reduction in the stearic to oleic acid ratio in leukemic cells: a possible marker for pregnancy. Blut 1985;50:349-354.

165. Eagle H, Foley GE. The cytotoxic action of carcinolytic agents in tissue culture. American Journal of Medicine 1956;21:739-749.

166. Wilson AP. Cytotoxicity and viability assays. In: Freshney RI, ed. Animal cell culture: a practical approach. 2nd ed. Oxford: Oxford University Press, 1992: 263-304.

167. Laerum F. Cytotoxic effects of six angiographic contrast media on human endothelium in culture. Acta Radiologica 1987;28(1):99-105.

168. Kim H, Park C, Lee YB, Yoo HS, Park JH, Lee W-Y. Effects of iodinated fatty acid ester on human hepatocellular carcinoma cells. Yonsei Medical Journal 1991;32(1):16-23.

169. Javitt NB. Hep G2 cells as a resource for metabolic studies: lipoproteins, cholesterol and bile acids. FASEB Journal 1990;4:161-168.

170. Guguen-Guillouzo C, Guillouzo A. Methods for preparation of adult and fetal hepatocytes. In: Guillouzo A, Guguen-Guillouzo C, ed.

187

Research in Isolated and Cultured Hepatocytes. John Libbey Eurotext Ltd/INSERM, 1986: 1-12.

171.   Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. Journal of Clinical Investigation 1973;52:2745-2756.

172.   Hamilton WJ, Mossman HW. Human Embryology. (4th ed.) London: MacMillan Press, 1976:342.

173.   Dhillon AP, Colombari R, Savage K, Scheuer PJ. An immunohistochemical study of the blood vessels within primary hepatocellular tumours. Liver 1992;12(5):311-318.

174.   Stansby GP. Isolation and purification of microvascular endothelium from human omentum for vascular graft seeding [M. S.]. University of Cambridge, 1992.

175.   Freshney RI, Paul J, Kane IM. Assay of anti-cancer drugs in tissue culture; conditions affecting their ability to incorporate Tritiated-Leucine after drug treatment. British Journal of Cancer 1975;31:89-99.

176.   Tygstrup N, Winkler N, Mellengaard K, et al. Determination of hepatic arterial blood flow and oxygen supply in man by clamping the hepatic artery during surgery. Journal of Clinical Investigation 1962;41:447.

177.   Bancroft JD, Stevens A, ed. Theory and Practice of Histological Techniques. 3rd edition ed. Edinburgh: Churchill Livingstone, 1990:220-222.

178.   Felton WL. A method for the identification of Lipiodol in tissue sections. Laboratory Investigation 1952;1:364-367.

179.   Arnold MM, Wallace AC, Kreel L, Li MKW. Demonstration of lipiodol in paraffin sections using a modified silver impregnation technique. American Journal of Clinical Pathology 1990;94:585-589.

180. Williams RA, Rode J, Dhillon AP, Jarvis LR, Skinner JM, Jamal O. Measuring S100 protein and neurone specific enolase in melanocytic tumours using video image analysis. Journal of Clinical Pathology 1986;39:1096-1098.

181. Fraser AG, Debnam ES, Dhillon AP, Pounder RE. Gastric epithelial cell proliferation after dosing with anti-secretory drugs: the effect of variation in two strains of rat. European Journal of Gastroenterology and Hepatology 1992;4:651-656.

182. Johnson PJ, Williams R. Cirrhosis and the aetiology of hepatocellular carcinoma. Journal of Hepatology 1987;4(1):140-147.

183. Sonoda T, Shirabe K, Takenaka K, Kanematsu T, Yasumori K, Sugimachi K. Angiographically undetected small hepatocellular carcinoma: Clinicopathological characteristics, follow-up and treatment. Hepatology 1989;10(6):1003-1007.

184. Motohashi I, Okudaira M, Takai T, Kaneko S, Ikeda N. Morphological differences between hepatocellular carcinoma and hepatocellular carcinoma-like lesions. Hepatology 1992;16:118-126.

185. Ferrell L, Wright T, Lake J, Roberts J, Ascher N. Incidence and diagnostic features of macro-regenerative nodules vs. small hepatocellular carcinoma in cirrhotic livers. Hepatology 1992;16:1372-1381.

186. Kurzawinski TR, Davidson BR, Bhattacharya S, Burroughs AK, Rolles K. Liver transplantation and primary liver tumours. Gut 1993;34(Supplement 1):S44.

187. Bhattacharya S, Davidson BR. Lipiodol computed tomography should be part of pre-operative assessment for liver transplantation. Journal of Hepatology 1994;20(2):310.

188. Pugh RNH, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices.

British Journal of Surgery 1973;60:646-649.

189. National Radiological Protection Board. Guidance notes for the protection of persons against ionising radiations arising from medical and dental use. London: Her Majesty's Stationery Office, 1988

190. Bourguet P, Raoul JL, Guyader D, et al. Randomised controlled trial of chemo-embolization vs. intra-arterial injection of 131 I-labelled iodized oil (Lipiocis) in the treatment of hepatocellular carcinoma. European Journal of Nuclear Medicine 1994;21 (Supplement)(10):S 52.

191. Kasugai H, Kojima J, Tatsuta M, et al. Treatment of hepatocellular carcinoma by transcatheter arterial embolisation combned with intra-arterial infusion of a mixture of cisplatin and ethiodized oil. Gastroenterology 1989;97:965-971.

192. Bronowicki JP, Nisand G, Altieri M, et al. Compared results of resection, orthotopic liver transplantation and transcatheter oily chemoembolisation in the treatment of Okuda's stage I hepatocellular carcinoma. Gastroenterology 1993;104(4 (Part 2;AASLD)):A881.

193. Strain AJ. Isolated hepatocytes: use in experimental and clinical hepatology. Gut 1994;35:433-436.

194. Pauli BU, Lee CL. Organ preference of metastasis: the role of organ-specifically modulated endothelial cells. Laboratory Investigation 1988;58:379.

195. Augustin-Voss HG, Johnson RC, Pauli BU. Modulation of endothelial cell surface glycoconjugate expression by organ-derived biomatrices. Experimental Cell research 1991;192:346-351.

196. Sato Y, Ochiya T, Yasuda Y, Matsubara K. A new three-dimensional culture system for hepatocytes using reticulated polyurethane. Hepatology 1994;19:1023-1028.

197. Minuth WW, Stockl G, Kloth S, Dermietzel R. Construction of an apparatus for perfusion of cell cultures which enables in vitro

experiments under organotypic conditions. European Journal of Cell Biology 1992;57(1):132-137.

198.   Eriksson LC, Andersson GN. Membrane biochemistry and chemical hepatocarcinogenesis. Critical reviews in Biochemistry and Molecular Biology 1992;27(1-2):1-55.

199.   Kan Z, Sato M, Ivancev K, et al. Distribution and effect of iodised poppyseed oil in the liver after hepatic artery embolisation: Experimental study in several animal species. Radiology 1993;186:861-866.

200.   Mallett SV, Cox DJ. Thromboelastography. British journal of Anaesthesia 1992;69(3):307-313.

201.   Gregoriadis G. Targeting of drugs: implications in medicine. Lancet 1981;2(8240):241-246.

202.   Mudan S, Loizidou M, Cooper AJ, Rew DA, Taylor I. Modulation of cytotoxic drug accumulation into human tumour cell lines and explanted cultures by essential fatty acids. Proceedings of the British Association of Surgical Oncology, 47th Scientific Meeting. London: The Royal College of Surgeons of England, 1993: 55.

Presentations and Publications

Abstracts of presentations

Bhattacharya S, Bradley NJ, Dhillon AP, Fuller BJ, Winslet MC, Hobbs KEF. Do liver tumour cells take up iodised oil? An in vitro study. Gut 1993; 34 (Supplement 4): S6

Bhattacharya S, Datta Gupta S, Dhillon AP, Bradley NJ, Winslet MC, Hobbs KEF. The effect of iodised oil (Lipiodol) on liver tumour and endothelial cells. Hepatology 1993; 18 (4, Part 2): 181A [Proceedings of the 1993 meeting of the American Association for the Study of Liver Disease (AASLD)]

Bhattacharya S, Novell JR, Al-Mufti R, Winslet MC, Hobbs KEF. Intra-arterial Lipiodol-Epirubicin in the management of hepatocellular carcinoma. Proceedings of the British Association of Surgical Oncology, 21st Anniversary Meeting, 1993, page 34 (published by the Royal College of Surgeons of England)

Bhattacharya S, Dusheiko G, Hilson A, Dick R, Hobbs K. Lipiodol-Epirubicin versus Lipiodol-^{131}Iodine for unresectable Hepatoma: A prospective randomised study. Proceedings of the Inaugural World Congress of the International Hepato-Pancreato-Biliary Association, Boston, Massachusetts, May 1994, page 6

Review article

Bhattacharya S, Novell JR, Winslet MC, Hobbs KEF. Iodised oil and the management of hepatocellular carcinoma. British Journal of Surgery 1994; 81(11): 1563-1571

Original articles

Bhattacharya S, Novell JR, Dusheiko GM, Hilson AJW, Dick R, Hobbs KEF. Epirubicin-Lipiodol chemotherapy versus ^{131}Iodine-Lipiodol radiotherapy in the treatment of unresectable hepatocellular carcinoma (Submitted to 'Cancer', and awaiting acceptance)

192

Bhattacharya S, Dhillon AP, Winslet MC, Davidson BR, Shukla N, Al-Mufti R, Datta Gupta S, Bradley NJ, Hobbs KEF. Human liver cancer cells and endothelial cells incorporate iodised oil (Submitted to 'Nature' and awaiting acceptance)

Letters

Bhattacharya S, Davidson BR. Lipiodol computed tomography should be part of pre-operative assessment for liver transplantation. Journal of Hepatology 1994; 20(2): 310

Appendix 1

Reagents used

Collagenase (Boehringer Mannheim)
Derived from *Achromobacter iophagus*
20 U/mg enzyme protein

Dulbecco's Modified Eagle's Medium (ICN Flow)
Constituents include $CaCl_2$, KCl, $MgSO_4$, NaCl, $NaHCO_3$, NaH_2PO_4, D-Glucose, Aminopterin, Hypoxanthine, Thymidine, Amino acids and Vitamins

Endothelial Cell Growth Supplement (Sigma)
Derived from bovine neural tissue
Lyophilised

Foetal calf serum (Gibco)
Gamma irradiated; screened for viruses and mycoplasma

Gentamicin (Sigma)
10 mg/ml

L-Glutamine (Sigma)
200 mM in 20 ml sterile water

Heparin (Sigma)
Derived from porcine intestinal mucosa
Sodium salt; Activity 170 USP units/mg

HEPES buffer (Sigma)
Prepared from HEPES free acid (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]) and sterile water

Medium 199
Constituents include $CaCl_2$, KCl, KH_2PO_4, $MgSO_4$, NaCl, NaH_2PO_4, D-Glucose, Adenine sulphate, Adenosine triphosphate, Cholesterol, Deoxyribose, Glutathione, Guanine, Hypoxanthine, Ribose, Sodium acetate, Thymine, Tween 80, Uracil, Xanthine, Amino acids and

Vitamins

Newborn calf serum (Gibco)
Screened for viruses and mycoplasma

Penicillin - Streptomycin (Sigma)
10,000 units Penicillin and 10 mg Streptomycin per ml in 0.9% saline

Phosphate-buffered saline (Gibco)
Contains KCl (0.2 g/l), KH_2PO_4 (0.2 g/l), NaCl (8 g/l), Na_2HPO_4 (1.15 g/l)

Trypsin EDTA (Sigma)
0.5 units Trypsin and 180 μg EDTA/ml

Appendix 2

Computer-assisted image analysis data

I. Human umbilical vein endothelial cells

Lipiodol concentration (vol/vol)	Duration of exposure (hours)	Number of fields assessed	Integrated optical density per cell (arbitrary units): Mean ± SD
0%	4	10	16.4 ± 9.7
1%	4	10	75.0 ± 69.3
2%	4	10	64.1 ± 25.7
4%	4	10	112.8 ± 48.3
0%	8	10	7.4 ± 2.9
1%	8	10	18.9 ± 9.9
2%	8	10	27.1 ± 12.8
4%	8	10	69.2 ± 16.9
0%	24	10	6.5 ± 5.6
1%	24	10	51.3 ± 16.4
2%	24	10	35.3 ± 17.3
4%	24	10	191.4 ± 74.8
0%	32	10	13.9 ± 8.3
1%	32	10	25.2 ± 13.2
2%	32	10	82.8 ± 45.9
4%	32	10	130.5 ± 51.7

II. HepG2 cells

Lipiodol concentration (vol/vol)	Duration of exposure (hours)	Number of fields assessed	Integrated optical density per cell (arbitrary units): Mean $\pm$ SD
0%	4	10	35.5 $\pm$ 14.6
1%	4	10	61.2 $\pm$ 11.4
2%	4	10	74 $\pm$ 39.5
4%	4	10	79.7 $\pm$ 35.1
0%	8	10	17.9 $\pm$ 11.2
1%	8	10	64.7 $\pm$ 25.9
2%	8	10	62.7 $\pm$ 17.7
4%	8	10	142.6 $\pm$ 66.7
0%	24	10	24.16 $\pm$ 9.6
1%	24	10	57.1 $\pm$ 25.6
2%	24	10	117.4 $\pm$ 34.4
4%	24	10	118.6 $\pm$ 54.2
0%	32	10	32.5 $\pm$ 12.1
1%	32	10	85.5 $\pm$ 25.5
2%	32	10	152.7 $\pm$ 58.1
4%	32	10	126.8 $\pm$ 52.1

Appendix 3

Patients who underwent Lipiodol angiography
prior to orthotopic liver transplantation

[Key: PSC   Primary sclerosing cholangitis

          PBC   Primary biliary cirrhosis

          CAH   Chronic active hepatitis

          HCV   Hepatitis C virus

          HBV   Hepatitis B virus

          MRN   Macro-regenerative nodule]

No.	Date of OLT	Indication for OLT	Foci of Lipiodol retention	Histology
1	30.07.93	PSC	No	
2	10.10.93	Alcohol	No	
3	20.10.93	Cryptogenic	No	
4	12.11.93	PSC	No	
5	23.11.93	HCV	No	
6	12.12.93	Alcohol	4 nodules	4 HCCs
7	01.01.94	PSC	No	
8	04.01.94	HCV	No	
9	13.01.94	HCV	1 nodule	1 MRN
10	04.02.94	PSC	No	
11	10.02.94	HCV	No	
12	12.02.94	HCV	No	
13	28.02.94	PBC	No	
14	07.04.94	PSC	No	
15	16.04.94	HCV	No	
16	17.04.94	HCV	No	
17	26.04.94	PBC	No	
18	02.05.94	Alcohol	No	
19	09.05.94	HCV	No	
20	24.05.94	PBC	No	
21	24.05.94	HCV	No	

Appendix 3   continued

No.	Date of OLT	Indication for OLT	Foci of Lipiodol retention	Histology
22	07.06.94	Alcohol	No	
23	26.06.94	Cryptogenic	No	
24	05.07.94	Alcohol	No	
25	19.07.94	CAH	No	
26	09.08.94	CAH	No	
27	17.08.94	HCV	No	
28	01.09.94	Alcohol	No	
29	06.09.94	HCC	5 nodules	5 HCCs
30	12.09.94	Alcohol + HBV	6 nodules	6 HCCs
31	19.09.94	Alcohol	No	
32	30.09.94	Alcohol	No	
33	03.10.94	PBC	No	
34	13.10.94	HBV	No	
35	19.10.94	HCV	No	
36	25.10.94	HCV	No	
37	26.10.94	PBC	No	

Appendix 4

WHO performance scale

Grade 0   Normal activity

Grade 1   Symptoms are present but activities are almost normal

Grade 2   Confined to bed for < 50% of each day

Grade 3   Confined to bed for > 50% but < 100% of each day

Grade 4   Incapable of rising

Appendix 5

Child's criteria for grading severity of liver disease
as modified by Pugh et al (188)

Clinical & biochemical measurements	Points 1	Points 2	Points 3
Encephalopathy	None	1 & 2	3 & 4
Ascites	Absent	Slight	Moderate
Bilirubin (mg / l)	10 - 20	21 - 30	> 30
Albumin (g / l)	> 35	28 - 35	< 28
Prothrombin time (seconds prolonged)	1 - 4	4 - 6	> 6

Grade A: 5 - 6 points
Grade B: 7 - 9 points
Grade C: 9 - 15 points

Clinical report form for patients in the prospective randomised study

ROYAL FREE HOSPITAL

Departments of Surgery, Medicine, Radiology & Nuclear Medicine

Clinical Report Form

Lipiodol ^{131}I _or_ Lipiodol Epirubicin

for

Unresectable Primary Hepatocellular Carcinoma

Lipiodol ^{131}I / Lipiodol Epirubicin for unresectable HCC

Check list for admitting HO / SHO:

[]   FBC

[]   U & E

[]   LFT

[]   AFP

[]   Viral markers for HBV & HCV

[]   CXR

[]   Abdo Ultrasound

[]   Abdo CT

[]   Coeliac and SMA angiogram

[]   Chest CT

[]   Bone scan

[]   Liver biopsy (Trucut/FNAC - confirm report with lab)

[]   Surgical opinion

[]   Informed consent prior to randomisation

[]   Colloid scan of liver for ^{131}I cases only (contact Medical Physics)

To do:

[]   Book X-ray Special Suite; Inform Dr Dick

[]   Inform Dr Hilson (for all ^{131}I cases)

[]   Order medication (Epirubicin Lipiodol from Sterile Pharmacy; ^{131}I Lipiodol through Medical Physics)

[]   Arrange radiation-shielded room on Moore Ward

[]   Inform Mr Bhattacharya on Bleep 840 (or Dr Dusheiko or Prof Hobbs)

Lipiodol ^{131}I / Lipiodol Epirubicin for unresectable HCC

To do (post-treatment):

- FBC, U&E, LFT on alternate days

- Liver SPECT at 48 hours (^{131}I cases only)

- Medical Physics to confirm safety of discharging patient (^{131}I cases only)

- Liver CT at 7-10 days

- Arrange follow up

PATIENT DATA SHEET   Page 1

All dates dd.mm.yy

ENROLMENT DATA

DATE OF ENTRY INTO TRIAL	
SURNAME	
NAME	
HOSPITAL NUMBER	
ADDRESS	
AGE IN YEARS (DATE OF BIRTH)	
SEX	**MALE / FEMALE**
THERAPY SELECTED	**LIP - EPIRUBICIN / LIPIODOL ^{131}I**

ELIGIBILITY CHECK LIST:
• **HCC confirmed**
 	
• **Tumour confined to liver**
 	
• **Tumour unresectable**
 	
• **Child's grade A or B, with**
   acceptable liver function
   (Bilirubin < 100, Albumin > 30)
 	
• **Patent Portal Vein**
 	
• **WHO performance status 0 - 3**
 	
• **Written informed consent**
	[ ] FBC
	[ ] U & E
	[ ] LFT
	[ ] AFP
	[ ] Viral markers for HBV & HCV
	[ ] CXR
	[ ] Abdo Ultrasound
	[ ] Abdo CT
	[ ] Coeliac and SMA angiogram
	[ ] Chest CT
	[ ] Bone scan
	[ ] Liver biopsy (Trucut / FNAC)
	[ ] Surgical opinion
	[ ] Informed consent

PATIENT DATA SHEET   Page 2

PRE-TREATMENT EVALUATION

HISTORY Duration (in months) Symptoms: • Jaundice • Wt loss / Anorexia / Nausea / Lethargy • RUQ pain • Abdominal lump • Ascites • Other Known cirrhotic Previous surgery Previous chemotherapy Previous radiotherapy	 Yes / No Yes / No Yes / No Yes / No Yes / No Yes / No If Yes: HBV / HCV / Alcohol / Other Yes / No Yes / No Yes / No
EXAMINATION Cachexia Oedema Jaundice Hepatomegaly Splenomegaly Ascites	 Yes / No Yes / No Yes / No Yes / No Yes / No Yes / No
Liver CT report	
Splanchnic angiogram report	
Liver biopsy positive? Biopsy report	 Yes / No
Summary of diagnosis: Tumour Lobes involved Okuda Stage Underlying chronic liver disease	 Solitary / Multifocal Right / Left / Both I / II / III Yes / No

Lipiodol 131I / Lipiodol Epirubicin for unresectable HCC

PATIENT DATA SHEET   Page 3

DRUG THERAPY

DATE OF TREATMENT:	
TREATMENT EPISODE NUMBER:	1 / 2 / 3 / 4
DRUG:	Epirubicin / Iodine 131
DOSE (mg of Epirubicin or MBq of 131I):	
TREATMENT MODIFIED?	Yes / No (If Yes, reason)
TREATMENT DELAYED?	Yes / No (If Yes, reason)
DURATION OF HOSPITAL STAY:	Days
OUTCOME OF HOSPITAL ADMISSION:	Discharged / Died

ADVERSE EFFECTS

Nature of complication	Onset date	Stop date	Duration	Relation to drug (Y / N / P) *	Treatment	Outcome**

*   Relation: Y = Related ; N = Unrelated ; P = Possible relation
** Outcome: 1 = Resolved ; 2 = Improved ; 3 = Unchanged ; 4 = Worse ; 5 = Fatal

PATIENT DATA SHEET **Page 4**

HAEMATOLOGY AND BIOCHEMISTRY

	Pre-treatment	7 days	30 days	
Hb (g/dl)				
WBC (x 10^9/ l)				
Platelets (x 10^9/ l)				
Urea (mmol/ l)				
Creatinine (umol/ l)				
Na (mmol/ l)				
K (mmol/ l)				
Cl (mmol/ l)				
Bilirubin (umol/ l)				
Albumin (g/ dl)				
AST (IU/ l)				
ALT (IU/ l)				
Alk Phos (IU/ l)				
INR				
HBsAg				
HBeAg				
Anti HCV				
AFP				

FOLLOW UP; RESPONSE TO THERAPY

Initial localisation of Lipiodol: • 48 hour SPECT (^{131}I cases only) • 10th day CT	Uptake Good / Moderate / Poor Uptake Good / Moderate / Poor
Follow up comments; Further investigations	
Overall response for this course	[ ] Complete response [ ] Partial response [ ] No change [ ] Progressive disease [ ] Not evaluable

PATIENT DATA SHEET   Page 5

DEATH WITHIN 4 WEEKS OF TREATMENT

Cause of death	
Death related to disease progression Death related to therapy	Yes / No / Possible Yes / No / Possible If Yes or Possible, explain

CONCLUSION SUMMARY (PATIENT COMES OFF THE STUDY)

Further courses of therapy	Yes / No
If treatment discontinued - reason(s)	[ ] Death related to treatment (see above) [ ] Death from unrelated reasons [ ] Loss to follow up [ ] Patient refusal [ ] Toxicity of therapy [ ] Disease progression
Status at end of follow-up	[ ] ALIVE [ ] DEAD [ ] LOST TO FOLLOW UP

Total duration of survival
     recorded after first course of
     therapy
	months

If dead, cause of death	[ ] Disease progression [ ] Drug toxicity [ ] Other

Appendix 7

Radiation dosimetry data (^{131}I recipients)

Not randomised

No	Tumour volume (cm³)	Activity injected (MBq)	Tumour dose (T) (in cGy)	Liver dose (L) (in cGy)	Lung dose (in cGy)	T/L ratio	Dose/Activity (cGy/MBq)
1	108	525	2068	199	266	10.4	3.9
2	15	475	7668	255	77	30.0	16.1
	22	632	4132	232	109	17.8	6.5
3	422	740	1042	150	248	6.9	1.4
	212	1315	3450	367	382	9.4	2.6
4	632	1164	1170	324	610	3.6	1.0
	822	280	176	92	93	1.9	0.6
5	1255	601	579	322	222	1.8	1.0
6	29	713	3257	347	377	9.4	4.6
7	45	720	2657	226	441	11.8	3.7
8	436	800	713	262	405	2.7	0.9
	136	992	365	372	91	1.0	0.4
9	38	961	2771	184	199	15.0	2.4
10	1200	247	40	102	66	0.4	0.2
11	1200	874	1734	255	727	6.8	2.0
12	934	800	500	350	696	1.4	0.6
13	72	743	4875	230	332	21.2	7.6
14	250	885	7642	446	506	17.1	8.6
15	64	853	3687	198	757	18.6	7.6
			1432			7.2	
			730			3.7	
			635			3.2	

Randomised

No	Tumour volume	Activity injected	Tumour dose	Liver dose	Lung dose	T/L ratio	Dose/Activity
16		900					
17		814					
18		930					

19		826					
20		1049					
21		969					
22		795					
23		944 808					
24		732 962 840					
25		841					
26		794 1047					

Appendix 8

Patients administered Lipiodol epirubicin and Lipiodol ^{131}I therapies

- Total patients: 95
- Numbers on this list do not represent the chronological order in which the patients were treated
- Numbers 1 to 67 (52 received epirubicin, 15 received ^{131}I) were not prospectively randomised
- Numbers 68 to 95 (17 received epirubicin, 11 received ^{131}I) were prospectively randomised

Non-randomised

No.	Age (yrs)	Child's grade of cirrhosis	Tumour stage (Okuda)	Treatment	Response	Survival (months)	Outcome
1	71	A	II	Epirubicin	NC	21	Dead
2	64	A	II	Epirubicin		2	Dead
3	66	A	II	Epirubicin	PD	9	Dead
4	45	C	III	Epirubicin		0.06	Dead
5	75	A	II	Epirubicin		2	Dead
6	68	B	II	Epirubicin	NC	9	Dead
7	66	B	II	Epirubicin	NC	30	Dead
8	33	B	II	Epirubicin		2	Dead
9	56	A	II	Epirubicin	NC	13	Dead
10	77	B	II	Epirubicin	PD	2	Dead
11	62	A	I	Epirubicin	PD	7	Dead
12	49	B	III	Epirubicin		1	Dead
13	39	B	II	Epirubicin		1	Dead
14	75	A	II	Epirubicin	PD	5	Dead
15	65	B	II	Epirubicin	NC	36	Dead
16	45	B	III	Epirubicin		1	Dead
17	56	B	II	Epirubicin		?	?
18	49	C	III	Epirubicin		0.03	Dead

19	63	C	III	Epirubicin		1	Dead
20	65	B	II	Epirubicin	PD	3	Dead
21	64	A	II	Epirubicin	NC	22	Dead
22	57	A	II	Epirubicin	NC	28	Dead
23	46		I	Epirubicin	PD	20	Dead
24	61	B	III	Epirubicin		2	Dead
25	50		I	Epirubicin	PD	10	Dead
26	61	A	II	Epirubicin		12	Dead
27	48	C	III	Epirubicin		1	Dead
28	69	A	II	Epirubicin	PD	4	Dead
29	66	A	II	Epirubicin		1	Dead
30	43	A	II	Epirubicin	NC	13	Dead
31	56	B	II	Epirubicin		0.4	Dead
32	81	A	II	Epirubicin	NC	18	Dead
33	74		II	Epirubicin	NC	14	Dead
34	78	B	III	Epirubicin		1	Dead
35	42		II	Epirubicin		20	Alive
36	52	A	II	Epirubicin		18	Alive
37	79		II	Epirubicin		0.3	Dead
38	67	B	III	Epirubicin		1	Dead
39	66	A	II	Epirubicin		14	Alive
40	45	B	III	Epirubicin		1	Dead
41	61	C	I	Epirubicin		12	Dead
42	57	B	III	Epirubicin	PD	2	Dead
43	60	B	III	Epirubicin		1	Dead
44	64	A	I	Epirubicin		10	Dead
45	76		I	Epirubicin		13	Alive
46	55		I	Epirubicin		13	Alive
47	66	B	II	Epirubicin	PD	5	Dead
48	63	C	III	Epirubicin		2	Dead
49	70	A	II	Epirubicin	NC	6	Dead
50	57	B	I	Epirubicin		?	?
51	58	A	II	Epirubicin	PD	5	Dead
52	56	C	III	Epirubicin		0.3	Dead
53	66	A	II	^{131}I	PR	3	Dead
54	63	A	I	^{131}I	PD	16	Alive

55	75	A	II	^{131}I	NC	4	Dead
56	62	B	II	^{131}I	PD	7	Dead
57	69	B	II	^{131}I	NC	1	Dead
58	68	A	I	^{131}I	NC	17	Dead
59	68	A	II	^{131}I		0.2	Alive
60	41	A	II	^{131}I	NC	19	Alive
61	55	A	II	^{131}I	PR	11	Dead
62	61	B	II	^{131}I	PD	4	Dead
63	48	A	II	^{131}I	NC	5	Alive
64	72		II	^{131}I	PD	2	Dead
65	71	B	II	^{131}I	NC	4	Alive
66	43	A	II	^{131}I	NC	4	Alive
67	63	A	I	^{131}I	NC	4	Alive

Prospectively randomised

No.	Age (yrs)	Grade of cirrhosis (Child-Pugh)	Tumour stage (Okuda)	Treatment	Response	Survival (months)	Outcome
68	69	A	II	Epirubicin	PD	4	Dead
69	69	A	I	^{131}I	NC	6	Dead
70	71	B	III	Epirubicin		1	Dead
71	20		II	^{131}I	PD	2	Alive
72	62	A	II	Epirubicin		2	Alive
73	63	B	II	^{131}I	PD	2	Dead
74	52	A	I	Epirubicin	PR	21	Alive
75	66	A	II	^{131}I	PR	12	Dead
76	62	B	I	Epirubicin	PD	3	Dead
77	66	B	II	Epirubicin	PD	4	Dead
78	61	A	I	^{131}I		6	Dead
79	35	A	I	Epirubicin	PD	4	Dead
80	59	B	I	Epirubicin	PR	12	Alive
81	65	A	II	^{131}I	PD	3	Dead
82	53	B	III	Epirubicin	PR	14	Alive
83	69	A	II	Epirubicin	NC	7	Dead

84	57	B	II	Epirubicin	NC	5	Dead
85	75		II	^{131}I		0.5	Dead
86	68		II	^{131}I	NC	6	Dead
87	61		II	^{131}I	PR	12	Alive
88	55	A	I	Epirubicin	NC	9	Dead
89	73	B	III	Epirubicin	PD	4	Dead
90	58		III	^{131}I		0.5	Dead
91	48	B	II	Epirubicin	NC	6	Alive
92	42	A	II	^{131}I	PD	5	Dead
93	60	A	II	Epirubicin	PR	7	Dead
94	64	A	I	Epirubicin	NC	8	Alive
95	63		III	Epirubicin	PD	3	Dead