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Acknowledgements—The support of the Anti-Cancer Council of Victoria is gratefully acknowledged.

Eur J Cancer, Vol. 29A, No. 12, pp. 1740–1745, 1993.
Printed in Great Britain

0959–8049/93 $6.00 + 0.00

A Hepatic Invasive Human Colorectal Xenograft Model

Susan A. Watson, Teresa M. Morris, David M. Crosbee and Jack D. Hardcastle

A hepatic invasive human colorectal xenograft model was derived in nude mice by selection through the liver of the parental cell line, C170. Following intraperitoneal injection, tumours selectively grew on the liver in > 80% of the animals within 15–20 days. The liver-invading xenograft line, renamed C170HM$_2$, had a significantly greater expression of the Lewis^x antigen compared to C170 (mean linear fluorescence per cell > 1000 compared with 500 for C170, $P < 0.02$). C170HM$_2$ had significantly elevated proliferation (when compared with C170) in the presence of epidermal ($P < 0.001$) and basic fibroblast growth factor ($P < 0.001$). C170HM$_2$ also mitogenically responded to type I collagen (derived from rat tails), unlike C170. C170HM$_2$ tumours when invading the liver expressed both interstitial collagenase and gelatinase activity at the invading edge.
Eur J Cancer, Vol. 29A, No. 12, pp. 1740–1745, 1993.

INTRODUCTION

METASTASIS OCCURS in greater than 60% of colorectal cancer patients with the major site of secondary tumour invasion being the liver [1, 2]. Secondary spread of malignant cells is one of the major causes of cancer fatality and can occur even before diagnosis and surgical resection of the original primary tumour [3].

The development of relevant *in vivo* models which allow therapeutic evaluation of potential antimetastatic agents is an important area of research. Experimental metastasis models exist in which colorectal tumour cells are administered intravenously (i.v.) [4–6]. Tumour cells have to survive in the circulation, arrest within an organ and outgrow and invade at a distant site. However, i.v. administration of colorectal tumour cells via the

tail vein results in lung and not hepatic metastases [4, 5]. Colorectal tumour cells may be injected into the spleen of nude mice (i.s.) [5, 7] but hepatic metastasis is dependant upon the intrinsic metastatic properties of the tumour cells and does not automatically occur [8].

Spontaneous metastasis studies are concerned with tumour invasion from the primary site and invasion and proliferation within the secondary site and may involve spread from a subcutaneous (s.c.) or an orthotopic site. Morikawa *et al.* [6] found human colon carcinoma cells did not form visceral metastases after s.c. implantation.

Orthotopic transplantation of colorectal tumour cells into the caecal wall [9, 10] results in the formation of hepatic metastases. However, large scale therapy determinations with such a model may be difficult due to the labour-intensive nature of the initiation (invasive surgery and injection into a vessel wall consisting of few cell layers) and low yield of metastatic liver tumours. In both experimental and spontaneous metastasis models it is possible to successively select metastasising tumour cells to generate sublines with increased metastatic properties [7]. In this study we have derived a selective liver-invading human colorectal xenograft in nude mice which is initiated by an intra-peritoneal (i.p.) injection. Thus it is a simple model to initiate, being both quick and requiring no invasive surgical technique and may allow large scale anti-metastatic therapy determinations.

MATERIALS AND METHODS

Animals

Male nude mice (6–8 weeks old) were utilised in all the *in vivo* studies (Harlan-Olac, Bicester, Oxford, U.K.). The animals were kept in sterile isolation at 30°C with food and water *ad libitum*.

Established cell lines

C170 was established from a poorly differentiated human colorectal primary tumour [11]. C170HM$_2$ is a sub line of C170 which was established by selecting liver-invading tumours. Tumours were selected five times, each time the liver-invasive tumour was enzymatically disaggregated with 0.5 mg/ml collagenase (Boehringer, Manheim, Germany) at 37°C for 20 min and 10^6 viable cells per mouse reinjected i.p.

Initiation of liver-invasive model with C170HM$_2$ cells

After five *in vivo* selections, the liver-invading tumours were enzymatically digested as described above, washed by centrifugation and grown as monolayer cell cultures in RPMI culture medium (Gibco, Paisley, U.K.) containing 10% heat-inactivated fetal calf serum (FCS, Gibco). The cells were grown at 37°C in a humidified incubator gassed with 5% CO_2.

C170HM$_2$ cells were harvested with 0.025% EDTA, resuspended in sterile 0.9% NaCl solution and 10^6 cells in a 1 ml volume were injected i.p. Experiments were terminated between days 30–40, at which time the liver-invasive tumours had grown (as determined by abdominal palpation), but the clinical condition of the animals was not compromised. U.K. Coordinating Committee for Cancer Research guidelines were adhered to throughout all the *in vivo* experimentation.

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Revised 25 Jan. 1993; accepted 18 Feb. 1993.

Tumour growth within the liver was evaluated by cross-sectional area measurements of tumour burden and liver weight. Histological analysis of liver-invading C170HM$_2$ tumours was also performed after fixation in formalin. Sections of the formalin fixed tissue were cut on a microtome at a thickness of 6 μm. The cells were then stained with haematoxylin and eosin, mounted and viewed microscopically.

Flow cytometric assessment of Lewis^x, carcinoembryonic antigen (CEA) and human cytokeratin expression on C170 and C170HM$_2$ cells

Cells from *in vivo* xenografts (prepared by enzymatic disaggregation) were examined by flow cytometry.

Measurement of Lewis^x and CEA expression was performed on fresh cells (as they are surface-associated antigens) and expression of human cytokeratin on pre-fixed cells (being an internal antigen). Fixation consisted of 10 min incubation with 1% paraformaldehyde at 4°C, followed by two washes by microcentrifugation and 10 min incubation with 70% ethanol at 4°C, again followed by two washes.

Lewis^x antigen expression was measured by staining with an anti-Lewis^x monoclonal antibody, 692-23 [12], CEA expression by an anti-CEA monoclonal antibody, NCR36 [13] and cytokeratin by an anti-cytokeratin monoclonal antibody recognising human cytokeratin 8 [11]. As all the monoclonals were of mouse origin, binding was detected with a rabbit anti-mouse fluoroscein isothiocyanate (FITC) labelled antibody (Dakopatts, High Wycombe, Bucks, U.K.). Mean green fluorescence per cell was measured on a Becton Dickinson flow cytometer, as described previously [14].

Enzyme-linked immunoabsorbant assay (ELISA) to detect secreted Lewis^x antigen

C170HM$_2$ and C170 cells were cultured in serum-free medium [Hams F12 (Gibco) in a 1:1 ratio with RPMI, containing 0.5% bovine serum albumin (BSA, Sigma)] for 24 h at 37°C. Control medium containing no cells was also incubated for the same time interval at 37°C. The supernatants were harvested and centrifuged to remove non-adherant cells.

Sera was also obtained from mice bearing liver invasive C170HM$_2$ tumours and non-tumour bearing nude mice.

Secreted Lewis^x antigen was measured by a sandwich ELISA technique as follows: the 692-23 antibody was coated on to 96 flat-bottom well microtitre plates (Becton-Dickinson, Oxnard, California, U.S.A.) overnight at 4°C at a protein concentration of 5 μg/ml. The plates were then washed with 0.9% NaCl solution containing 0.1% BSA twice and either cell culture supernatant, control medium or mouse sera were added to the wells. Patient sera known to be positive and negative for Lewis^x antigen expression (provided by L. Durrant) were used as controls. After washing, the wells were incubated with biotinylated 692-23 antibody (10 μg/ml), binding of which was detected with avidin immunoperoxidase (Dakopatts) and substrate (ABTS). In addition, binding of cell culture supernatant to the immobilised 692-23 antibody was detected with a well characterised anti-Lewis^x monoclonal antibody [15]. This antibody was provided in the form of ascites and used at a protein concentration range between 500 and 10 μg/ml. Detection of binding was the same as for biotinylated 692-23. Absorbance was measured at 405 nm.

Metalloproteinase expression

Interstitial collagenase expression was determined on frozen samples of liver-invading C170HM$_2$ tumours (quenched in

isopentane which had been precooled in liquid nitrogen) and stored at −70°C as described [16]. Gelatinase levels were measured by Dr Peter Brown, British Biotechnology, Oxford, U.K. on frozen tumour samples by zymography [17]. Briefly, tumour tissue was homogenised and loaded on to an acrylamide gel containing 10% v/v gelatin (12 mg/ml). Following electrophoresis the gelatin substrate present in the gel is degraded by the band of enzyme. When the gel is stained for gelatin (0.5% w/v Coomassie blue), the presence of the enzyme is revealed by a zone of substrate clearance.

In vitro proliferation of C170 and C170HM$_2$

The *in vitro* proliferation of C170 and C170HM$_2$ was measured in the presence of a number of growth factors and collagen type I: epidermal growth factor (EGF) was obtained from Sigma and used at a concentration range of 200–5 ng/ml. Fibroblast growth factor acidic and basic (FGFa, FGFb, Sigma) were used at a concentration range of 100–0.1 ng/ml. Liver and spleen tissue extracts were prepared by aseptically removing the relevant organs from a non-tumour-bearing nude mouse. The organs were mechanically minced through a 60 μm metal grid, in 10 ml of the serum-free medium previously described. The medium was aspirated and centrifuged to remove cellular debris. The protein content of the tissue extracts was measured by a protein assay kit (Pierce, Oud-Beijerland, Holland) and adjusted with serum-free medium to yield a concentration approximating to 1.0 mg/ml.

Type I collagen was prepared from rat tails. The tails were dissected and the tendons removed. Collagen was prepared according to the method of Bornstein *et al.* [18], lyophilised, weighed, reconstituted with serum-free medium and plated into 96 flat-bottomed well tissue culture plates (Flow Labs, Irvine, U.K.) in 25 μl aliquots and gelled with 25 μl serum-free medium giving a final concentration of 0.5% well.

C170 and C170HM$_2$ cells were harvested with 0.025% EDTA and plated into 96-well plates (in the case of collagen, directly on to the gel), at a concentration of 10^4 cells/well. After allowing for cell adherence, the growth factors/tissue extracts diluted in serum-free medium, were added directly to the cells (five replicates were performed per growth factor dilution). The cells were incubated for 48 h at 37°C before being pulsed with ^{75}[Se]selenomethionine (CIS, U.K., 0.1 μCi/well) for a further 18 h at 37°C. ^{75}[Se]selenomethionine uptake has been shown to correlate closely with direct cell counts [19]. The plates were then washed and associated radioactivity per well counted on a γ-counter.

The mean of the five replicates was calculated for each condition and results were expressed as a per cent of the untreated control.

Statistical analysis

All statistical analyses were performed using the Student's *t*-test.

RESULTS

Initiation of the C170HM$_2$ hepatic-invasive model

The human colorectal tumour line, C170 was injected i.p. into 10 nude mice, four of which developed liver tumours in addition to peritoneal growths. The liver-invasive tumours were enzymatically disaggregated and passaged through the liver four more times. This resulted in a cell line which selectively invaded the liver resulting in macroscopic lesions between days 30 and 40.

Assessment of the tumour burden was made by abdominal examination and palpation. If palpable lesions were present the experiment was terminated.

The liver invasive cells were expanded *in vitro* and denoted C170HM$_2$. To ensure the C170HM$_2$ cell line had maintained its liver-invasive properties, 10^6 cells were reintroduced i.p. into eight nude mice. After a period of 49 days the experiment was terminated and the liver tumour burden of the animals visually assessed by two observers. It was found that 100% of the animals had tumour within their livers but the degree of tumour growth varied between individual animals. In 75% of the animals, more than 40% of the liver had been overtaken with tumour, and in the remaining 25%, the tumour burden was between 5 and 10% of the liver.

The liver weights of age- and sex-matched nude mice were compared in non-tumour-bearing nude mice and mice bearing C170HM$_2$ tumours. The non-tumour-bearing livers from eight mice had a mean weight of 1.65 g (± 0.2 S.D.) and the tumour-bearing livers had a mean weight of 2.28 g (± 0.26 S.D.) which was significantly greater ($P < 0.001$).

To date, 150 animals have been injected i.p. with 10^6 C170HM$_2$ cells and liver lesions have formed in 81% of the animals. Peritoneal nodules grew in < 10% of the animals.

Histological examination of liver-invasive C170HM$_2$ xenografts

Histological analysis of consecutive sections through livers of animals injected i.p. with C170HM$_2$ cells revealed that all tumours were associated with the surface, indicating cells invaded via the liver capsule and were not blood/lymphatic borne metastases in that they did not arrive and invade from the circulation. However, blood-borne spread cannot be completely ruled out as it may occur in some instances.

Comparison of antigen expression on C170 and the hepatic invasive sub-line, C170HM$_2$

The antigen expression of C170HM$_2$ xenografts growing within the liver or s.c. was compared to the original xenograft, C170 growing s.c. All three xenografts were positively stained for human cytokeratin (mean linear fluorescence between 800 and 1400 per cell) yet did not express CEA (Fig. 1).

All cell lines expressed the Lewis^x antigen but this was significantly elevated on the intra-hepatic C170HM$_2$ xenograft ($P < 0.02$) when compared to the s.c. C170 xenograft (Fig. 1).

Lewis^x secretion by C170HM$_2$ during in vitro *growth*

An ELISA was performed to detect Lewis^x antigen secretion by C170HM$_2$ cells during *in vitro* culture by utilising an anti-

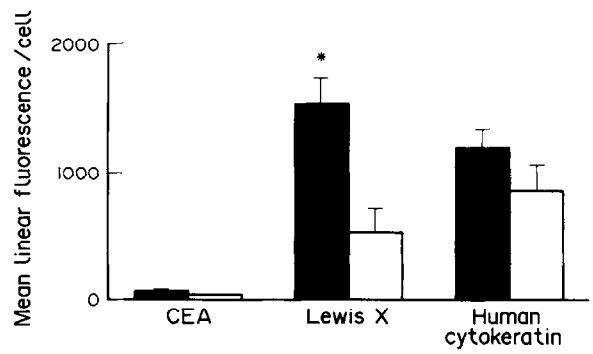

Fig. 1. Flow cytometrical analysis of Lewis^x, CEA and cytokeratin expression on ☐ s.c. C170 xenograft and ■ C170HM$_2$; liver invasive xenograft. Expressed as mean linear fluorescence (MLF) per cell. *$P < 0.02$, when compared to the expression on s.c. C170 xenografts.

Lewis^x antibody to detect antigen within the culture supernatant. The optical density (OD) of the culture medium from C170HM$_2$ growing exponentially detected with the anti Lewis^x antibody, 692–23 gave an absorbence value of 0.69 when used at a 1/2 dilution compared with an absorbance value of zero for culture medium alone. The positive control which was serum from a colorectal cancer patient gave an OD of 0.89 at a 1/2 dilution whereas a negative sera (again from a colorectal cancer patient) and culture medium from C170 cells gave an OD of 0 at all dilutions examined.

Detection of the Lewis^x antigen in C170HM$_2$ cell culture supernatant was compared utilising 692–23 and a well characterised anti-Lewis^x monoclonal antibody [15]. C170HM$_2$ culture supernatant at a 1/10 dilution gave an OD of 0.43 with 692–23 and an OD of 0.25 with the second anti-Lewis^x antibody (used at an equivalent protein concentration).

Sera from mice with liver-invading C170HM$_2$ tumours were also examined for the presence of the Lewis^x antigen and compared to serum from a non-tumour-bearing nude mouse. By detection with the 692–23 antibody, the OD of sera from tumour-bearing mice ranged from 0.123–0.238, whereas the sera from the non-tumour-bearing mouse gave an OD of 0.057.

This indicates that C170HM$_2$ cells have the ability to secrete Lewis^x antigen during *in vitro* and *in vivo* growth.

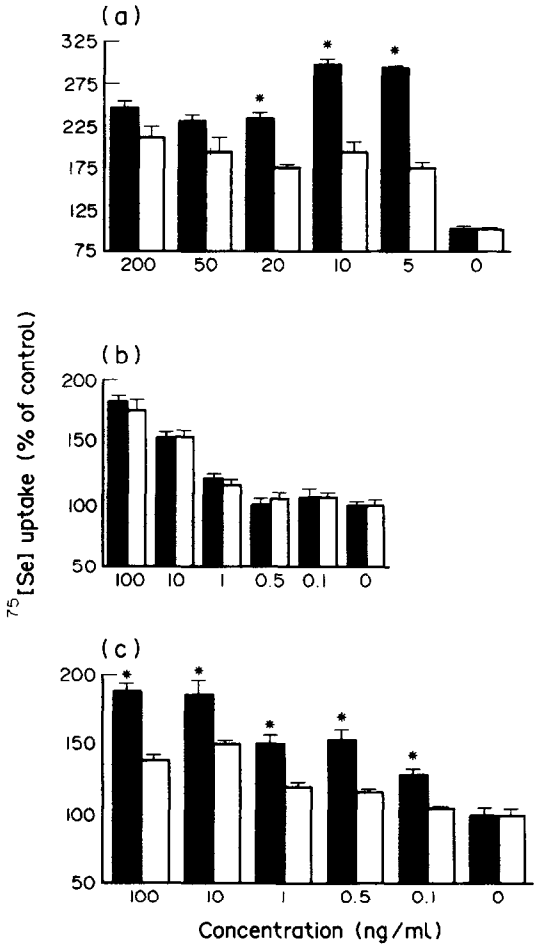

Fig. 2. The effect of (a) EGF (b) FGFa and (c) FGFb on the proliferation of □ C170 and ■ C170HM$_2$ as assessed by ^{75}[Se]-selenomethionine uptake (expressed as a % of the untreated control). *$P < 0.001$ from proliferation achieved with C170 cells.

Comparative effects of growth factors on the proliferation of C170 and C170HM$_2$ in vitro

Soluble organ extracts. The effects of soluble tissue extracts from the spleen and liver of non-tumour-bearing nude mice were examined on the *in vitro* proliferation of C170 and C170HM$_2$ cells as assessed by ^{75}[Se]selenomethionine uptake.

As compared with the proliferation in the serum-free medium control, both C170 and C170HM$_2$ had significantly increased label uptake in the presence of spleen tissue extract, ($P < 0.001$ for both cell lines) of 135% and 150% of the untreated control, respectively. Both cell lines also had enhanced label uptake in the presence of liver tissue extract. C170 cell proliferation was increased to 145% and C170HM$_2$ proliferation to 227% of the untreated control both of which were significant from the control ($P < 0.001$ for both cell lines). The label uptake achieved by C170HM$_2$ was also significantly elevated from that achieved with C170 ($P < 0.001$).

Type I collagen. Collagen did not significantly affect the proliferation of C170 but the proliferation of C170HM$_2$ was increased to 174% of the control which was significant ($P < 0.001$) from that of the medium control and C170. Attachment of the cells to type I collagen was assessed by direct cell counts and it was found that C170 and C170HM$_2$ cells bound to the collagen gel to an equal extent (results not shown).

EGF, acidic and basic FGF. The effect of EGF on the proliferation of C170 and C170HM$_2$ was compared (Fig. 2a shows the results from a typical experiment which has been repeated three times). Both cell lines responded significantly to EGF as assessed by an increase in label uptake at concentrations ranging from 200 to 5 ng/ml. However, at EGF concentrations of 20, 10 and 5 ng/ml, C170HM$_2$ showed a significantly greater proliferative response than C170 ($P < 0.001$).

Figure 2c shows the results obtained with basic FGF from a single typical experiment from a series of three performed. C170 had enhanced label uptake in the presence of basic FGF at concentrations from 100 to 0.5 ng/ml. C170HM$_2$ also had enhanced label uptake in the presence of basic FGF at concentrations from 100 to 0.1 ng/ml. However, at each FGF concentration the proliferation of C170HM$_2$ was significantly enhanced from that achieved with C170 ($p < 0.001$ at all FGFb concentrations). Figure 2b shows the effect of acidic FGF on the label uptake of C170 and C170HM$_2$ from a typical experiment of three performed. Both cell lines responded significantly to acidic FGF concentrations from 100 to 1ng/ml with each line responding to the same degree.

Collagenase expression by C170HM$_2$. Interstitial collagenase expression was measured by immunohistology and was found to be present at the invading edge of C170HM$_2$ xenografts within the liver. In addition, collagenase was also present within the tumour (associated with connective tissue).

Liver invading C170HM$_2$ tumour tissue had detectable levels of the 72 kD gelatinase in the tumour tissue but not in adjacent liver.

DISCUSSION

Existing models involving the metastatic spread of colorectal tumour cells to the liver allow detailed examination of the metastatic process [9]. Models initiated by intracaecal injections [9, 10], and intrasplenic injections [5, 7] have advantages in that intracaecal administration allows metastasis to be followed after

transplantation at an orthotopic site, and intrasplenic initiation allows an assessment of the intrinsic metastatic capacity of tumour cells. However, such models are surgically invasive and technically demanding and may not result in a high proportion of animals within a group having liver tumours within a set time scale. This may generate problems when trying to initiate large scale experiments for therapy determinations.

The model described in our study, C170HM$_2$ was selected through the liver of nude mice after an initial i.p. injection of the original cell line C170. As it is known that human tumour cell lines contain subpopulations of cells with variable metastatic capabilities [20] such a process allows selection of metastatic sublines, as shown by Morikawa *et al.* [7]. The resultant cell line, C170HM$_2$ was organ specific and liver tumours were formed via invasion of the liver surface in greater than 80% of mice, which is crucial for therapy determinations. Initiation of the model is quick to perform and is surgically non-invasive, requiring no anaesthesia. The tumours start to become visible within the liver between days 15 and 20 and termination occurs around day 40. Thus the experimental time period is short when compared to other metastatic models which is crucial when administering therapy, as efficacy evaluation is quick and long-term delivery of drugs is avoided.

Therapy can be administered from day 0 (allowing efficacy to be determined in an adjuvant setting) or from day 20 (efficacy determined in an advanced setting) allowing correlation with a potential clinical treatment of colorectal cancer patients. Termination of the model at day 40 resulted in > 80% tumour burden within the liver yet the animals remained in a good clinical condition.

End-point determinations of metastasis models are generally difficult to measure. Time to morbidity not only involves clinical compromisation of the animals but may not be due, in every case, to metastatic spread.

In the C170HM$_2$ model, at termination, subjective measurements can be performed by visual assessments and objective measurements by weighing the whole liver. Livers bearing C170HM$_2$ xenografts weigh significantly more than non-tumour-containing livers. In addition, as the invading cells produce Lewis^x antigen *in vivo* it is envisaged that serum antigen assessment may also yield a second objective assessment of end-point tumour burden.

The hepatic invasive model described is not a metastasis model as the initial invasion steps from the primary site are bypassed. However, it does allow evaluation of the end point and secondary growth of human colorectal tumour cells at the relevant site of spread. The tumour invades within the liver and the invading edge has been shown to be associated with interstitial collagenase and gelatinase activity which degrade connective tissue extracellular matrix and basement membrane, respectively and have been found in a wide variety of tumours [21]. It has been shown that in colorectal carcinomas, elevated collagenase production was associated with deeper invasion [22].

In the present study, selection of a liver-invasive phenotype was associated with an enhanced expression of the Lewis^x trisaccharide which was also secreted in a soluble form both *in vitro* and *in vivo*, indicating it was sialosylated [23]. It has been shown that preoperative levels of Lewis^x antigen increased with colon cancer progression in that 74% of Dukes D stage tumours had detectable antigen levels compared to 20% of Dukes A stage colon cancers [15]. In addition, it has been shown in a study by Aznavoorian *et al.* [24] that invasive cells from a human colon adenocarcinoma were highly sialosylated. As C170HM$_2$ may express elevated levels of sialosylated Lewis^x this may result in an enhanced ability to attach to the liver and thus invade. It was shown in the present study that C170HM$_2$ has a greater capacity to proliferate on collagen *in vitro* than C170 which may correlate with an enhanced adhesiveness.

Neither C170 or C170HM$_2$ expressed CEA which was possibly by virtue of them being derived from a poorly differentiated primary tumour.

C170HM$_2$ was shown to be more sensitive to the proliferation-enhancing effects of EGF and FGFb when compared to C170. In context with this it has been shown that EGF pretreated rat rhabdomyosarcoma cells when injected i.v. had an enhanced ability to colonise the lungs [25]. In addition, an antibody to the EGF receptor inhibited the spontaneous metastasis of a human melanoma cell line [26]. EGF induces a multitude of effects, in addition to mitogenically stimulating the cells, which may aid secondary spread. It was found to increase the membrane expression of the cell attachment proteins, fibronectin and laminin which have a major role on cell adhesiveness in a human breast cancer cell line, PMC42 [27]. In addition, EGF can control enzymes such as metalloproteinases [28] which play an important role in invasion and secondary spread.

FGFb is an insoluble growth factor bound into basement membranes [29] and known to be relevant in metastasis. Thus it is feasible that selection of cells with increased invasiveness led to a cell population with enhanced sensitivity to the mitogenic effects of FGFb.

The seed and soil hypothesis of metastasis is well described [8] and C170HM$_2$ has been shown to selectively invade the liver, despite being in contact with other organs in the peritoneal cavity. It was shown that soluble liver extract enhanced the growth of C170HM$_2$ to a greater extent than C170 indicating growth-promoting factors may play a role in the liver-specific invasion. It was noted by Nicholson and Dulski [30] that metastasis to specific organs was affected by soluble organ-derived factors and Rusciano *et al.* [31] found liver extracts promoted the growth of murine F9 embryonal carcinoma cells which preferentially colonise the liver upon i.v. injection via the tail vein. In addition to mitogenic effects, tissue-specific factors may affect local invasion by colon cancer cells as it was shown that partially purified liver extracts enhanced the ability of cells to penetrate a reconstituted basement membrane in a modified Boyden chamber [9]. In context with this a study by Nakajima *et al.* [32] revealed that factors in an organ environment may affect production and secretion of degradative enzymes by colon carcinoma cells *in vivo*.

The intrahepatic human colorectal xenograft, C170HM$_2$ should allow the study of invasion and proliferation of colorectal tumour cells at the secondary site of metastasis and also allow large experimental groups of tumour-bearing mice to be initiated for evaluation of potential anti-metastatic therapies.

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Acknowledgements—The authors would like to thank Dr Robert Hewitt for his advice and for staining the formalin-fixed sections. In addition, gratitude is expressed to Dr Lindy Durrant for the provision of the 692-23 monoclonal antibody, Dr Peter Brown for the measurements of gelatinase activity and Miss D. Milanowska for typing the script. Financial support was provided by a Medical Research Council Industrial Award, grant number G8802476CA and the Cancer Research Campaign, U.K.