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CHARACTERISATION OF THE VASCULATURE WITHIN A MURINE ADENOCARCINOMA GROWING IN DIFFERENT SITES TO EVALUATE THE POTENTIAL OF VASCULAR THERAPIES

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Numerous vaso-active agents can affect vasculature in experimental solid tumours growing subcutaneously (s.c.), but these models are unlikely to reflect the vasculature of metastatic disease in man. The present study describes a murine orthotopic colon tumour which metastasises to the liver. Morphology and vascular pattern of caecal tumours is similar to s.c. tumours. Vascular occlusion caused by intravenous (i.v.) noradrenaline (NA) (160 $\mu g kg^{-1}$) and hydralazine (HDZ) (10 $mg kg^{-1}$) was 32% and 59% respectively for the caecal tumours compared with 35% and 78% for s.c. tumours. Significant morphological differences were seen between liver metastases and systemic deposits produced by i.v. inoculation of tumour cells. Liver metastases following orthotopic transplantation contained functional vasculature but no significant occlusion was seen with NA or HDZ. The vascular development and morphological appearance of secondary disease resulting from orthotopic implantation suggests that this would be a useful model for the study of agents that act either by vascular or anti-angiogenic mechanism.

An ideal experimental animal model for cancer is one which mimics human disease in every respect. Relevant experimental models are needed for the study of potential anti-cancer therapies and particularly to evaluate new strategies for therapy, e.g. anti-angiogenesis (1). The primary target for cancer chemotherapy is metastatic disease with the majority of primary neoplasms being surgically excised. Widely used experimental models of large bowel cancer, e.g. rodent s.c. tumours, will be influenced by site of growth and may possess a microenvironment which is not clinically appropriate (2). The vascular network within, and surrounding tumour tissue will have a major impact upon the microenvironment and will not only influence growth and spread but is important for drug delivery. The present study investigates an orthotopic model of murine colon cancer with a view to developing a more clinically relevant system in which to investigate vascular development. Previous studies using subcutaneous (s.c.) tumours have demonstrated drug potentiation when a dual treatment of vaso-active agent and chemotherapeutic drug are given (3–5). Vascular occlusion is associated with such treatments of this site (6). The present study attempts to assess the clinical relevance of this approach by using tumour deposits at more appropriate anatomical sites.

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Material and Methods

Animals. Pure strain NMRI male mice aged 6–8 weeks from an inbred colony (B & K Universal Limited, Hull, England) were used. Mice received CRM diet (Labsure, Croydon, England) and water ad libitum. All animal procedures were carried out under a project licence issued by the Home Office, London.

Tumour system. A moderately rapidly growing, well-differentiated, tubular adenocarcinoma (MAC15) origi-

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nally induced in the colon of NMRI mice by weekly administration of 1, 2 dimethylhydrazine (7) was used. The MAC15 tumour was maintained by weekly s.c. transplantation. Studies were performed when the tumours reached a diameter of approximately 1–1.5 cm. A MAC15A ascites tumour originally derived by intraperitoneal implantation of a MAC15 tumour piece (8) and subsequently re-developed was used for intravenous (i.v.) model studies.

Intravenous model. 1×10^6 MAC15A ascites tumour cells were injected i.v. into the tail vein. Systemic deposits were visible, particularly in the lung, approximately 18 days after injection.

Orthotopic tumour implantation. A donor MAC15 s.c. tumour was resected aseptically and placed in a Petri dish containing sterile saline, penicillin and streptomycin. Viable tumour tissue was cut into pieces approximately 1 mm^3. Mice were anaesthetised with Metofane inhalation anaesthetic (C-VET, Bury St Edmunds, England). A 1–1.5 cm incision was made in the shaven abdomen, left of the mid-line, and the caecum was gently externalised. Transplantation of a piece of MAC15 tumour was achieved either by using a small trocar inserted into the caecal wall or by placing a piece of tumour on the surface of the caecum and covering this with a small disc of cellulose acetate membrane (Millipore UK Ltd, Watford, England, Type RA pore size 1–2 μm). The implant was fixed into position with Histoacryl blue tissue adhesive (Davis & Geck, Cyanamid of Great Britain, Gosport, Hampshire, England). The caecum was returned carefully to its normal position within the abdomen and then the abdominal wall was sutured using 10 mm precision point needles, 6/0 prolene (Ethicon Ltd., Edinburgh, U.K.). The skin was closed using autoclips (Clay Adams, Division of Becton Dickinson & Co., New Jersey, USA). The clips were removed once sufficient wound healing had occurred; usually one week after surgery. Studies were performed when the tumours were approximately 1.5–2 cm in diameter.

Hoechst-33342 *study* (H33342). (bisBenzimide, Sigma, Poole, Dorset, England). H33342 dissolved in sterile saline was administered at 40 mg kg^{-1} (volume 5 $mlkg^{-1}$) i.v. (9). One minute after injection the mouse was killed by cervical dislocation, dissected and the tumour and liver resected. Tissues were wrapped in aluminium foil and immediately immersed into liquid nitrogen for subsequent frozen sectioning.

Relative perfusion analysis. Five μm frozen sections were cut on a cryostat (Bright Instrument Co. Ltd., Huntingdon, England). These were viewed at × 100 magnification on a light microscope using incident UV illumination. Perfusion was observed as fluorescence and a graticule used to calculate percentage relative perfusion as the number of fluorescent squares divided by the total number of squares × 100. A minimum of 5 sections per tissue was examined and 5 fields were counted per section.

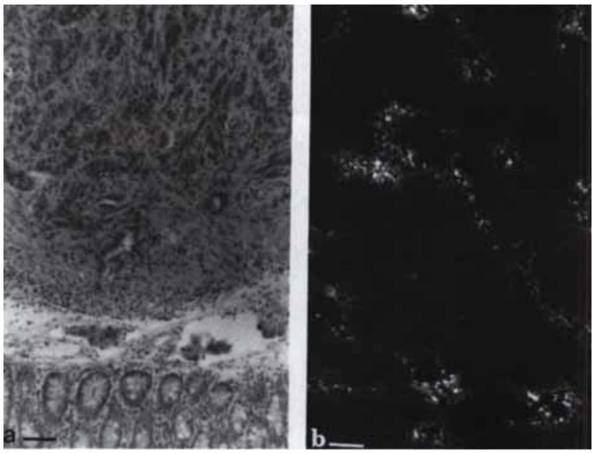

Fig. 1. Morphological appearance of MAC15 growing in the caecum of NMRI mice. a) Haematoxylin and eosin (bar 60 μm) b) Visualisation of blood vasculature in a frozen section by Hoechst 33342 (bar 25 μm).

Histological analysis. Tumour, liver and lung tissue was processed for histological analysis. Five μm paraffin sections were prepared and stained either in haematoxylin and eosin (H + E) or with a Gomori's trichrome stain to facilitate easier identification of stromal and epithelial components.

Immunohistochemistry. An indirect immunohistochemical technique was performed on both 5 μm cryostat and paraffin sections to observe the CD31 antigen (PECAM-1) which is situated within the cell membrane of endothelial cells. Following appropriate fixation or trypsinisation stages, sections underwent an initial incubation with blocking serum (whole rabbit serum for 30 min), sections were then washed in a bath of Tris buffered saline (TBS) pH 7.6. The primary antibody was purified anti-mouse CD31 (Pharmingen, Cambridge Bioscience, Cambridge, England). Stock antibody was optimally diluted 1:200 with TBS before application to tissue sections for 1 h. Sections were then washed in TBS for 10 min. The secondary antibody was biotinylated affinity isolated rabbit immunoglobulins to rat immunoglobulins (Dako Ltd., High Wycombe, Bucks., England). The stock solution was optimally diluted 1:300 with TBS before application to tissue sections for 30 min. Sections were then washed in TBS for 10 min and incubated with a complex of avidin and biotinylated horseradish peroxidase for 30 min (ABC complex/HRP, Dako Ltd., High Wycombe, Bucks., England). Following a brief wash in TBS sections were incubated with a peroxidase substrate solution (3,3′-diaminobenzidine Tetrahydrochloride, DAB, (BDH Laboratory Supplies, Poole, England). Slides were transferred to a copper sulphate solution for 5 min before being counter-stained with haematoxylin, dehydrated and mounted.

Experimentation with vaso-active drugs. Mice bearing the MAC15 s.c. or orthotopic tumours were each divided

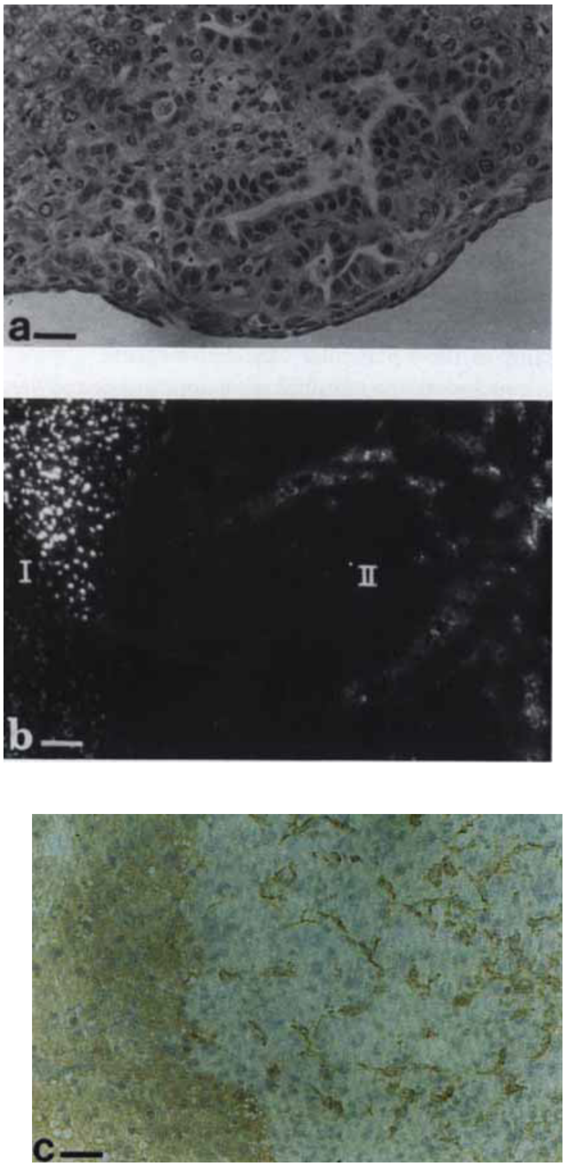

Fig. 2. MAC15 liver metastases. a) Gomori's trichrome stain (bar 25 μm); b) Hoechst 33342 fluorescence in a frozen section, I denotes liver tissue, II denotes a metastatic deposit (bar 60 μm); c) CD31 immunohistochemical stain (bar 25 μm).

into one control and two treatment groups, each group containing a minimum of 4 animals. The treatment groups received either HDZ (10 $mgkg^{-1}$) or NA (160 μgkg^{-1}) i.v. H33342 was administered 9 min and 4 min after treatment for HDZ and NA respectively. Timing and dose levels for these s.c. treatments was based on previous studies in this laboratory (6, 10). Mice were killed by cervical dislocation one minute post H33342. Tumour and liver tissue was collected, and the morphology and percentage relative perfusion analysed as described previously. The control animals received H33342 alone. Vasculature shutdown was calculated by comparison of treated to control tumours.

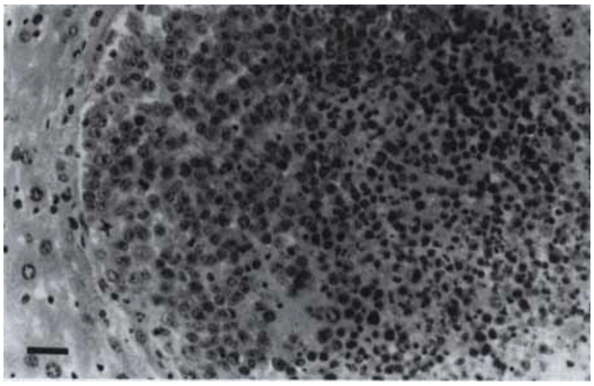

Fig. 3. Histological appearance of tumour deposit in the liver of an NMRI mouse following i.v. inoculation of MAC15A cells. (H + E stain, bar 25 μm).

Table

Effect of vaso-active agents on % vascular shutdown in MAC15 tumours growing in two different sites

	Percentage shutdown	
Site	Noradrenaline 160 μgkg^{-1}	Hydralazine 10 $mgkg^{-1}$
Subcutaneous	31	82
	43	71
	34	88
	32	87
	–	61
Mean	35 ± 5	78 ± 12
Orthotopic caecum	22	51
	27	60
	48	55
	45	68
	20	63
Mean	32 ± 13	59 ± 7

Data represent mean values for individual tumours.
Mean values for groups of treatments are given ±1 SD.

Results

Morphological studies. The orthotopic implantation of MAC15 as a solid tumour fragment resulted in localised aggressive tumour growth infiltrating the caecum. The morphological appearance of the tumours at this site was of a well-differentiated glandular adenocarcinoma with both stromal and functional vascular structure (Fig. 1a and b). They were identical in appearance to the s.c. donor tumours. Large tumours growing in the orthotopic site developed necrotic centres with a well-vascularised periphery as did s.c. tumours. Large palpable tumours were apparent from between 6 to 8 weeks after caecal implantation and at this stage both liver and lung metastases were common. Histological examination of liver deposits indicated well-differentiated tumours with clear glandular and stromal components (Fig. 2a). The fluorescent dye H33342 demonstrated a functional network of vessels (Fig. 2b) and immunohistochemical staining for CD31 antigen indicated extensive endothelial development (Fig. 2c). This metastatic spread was unlike that observed following i.v. inoculation of MAC15A cells where the major site of secondary tumour growth was the lungs with occasional deposits being observed within the liver. Histological analysis of these deposits showed an undifferentiated state with tumour cells forming an unstructured mass with no obvious stromal or vascular involvement (Fig. 3).

The effects of vaso-active agents. The influence of NA and HDZ on the relative perfusion of MAC15 tumours growing in a s.c. and orthotopic site is presented in the Table. Data describe percentage vascular shutdown after comparison of treated tumours with control tumours. Control data show no significant difference ($p = 0.20$) in the relative perfusion of viable untreated tumour within these sites. There appeared to be no difference in the effectiveness of NA to shut down the vessels in the two different sites ($p = 0.73$), whereas HDZ appeared slightly more effective at shutting down vasculature in the s.c. tumour. HDZ nevertheless still caused major effects in the orthotopic site.The effect of HDZ in these two sites was significantly different ($p = 0.015$). No significant occlusion of vessels was demonstrated in the hepatic metastases, produced following orthotopic transplantation, by either agent. The relative perfusion of these deposits was significantly different ($p = 0.02$), being 70% of that observed in the s.c. or orthotopic sites.

Discussion

The present study demonstrated that the murine adenocarcinoma MAC15 can be successfully grown following two orthotopic implantation techniques, producing an aggressive tumour which retains the morphological characteristics of the donor tumour and which is metastatic to the liver and lungs. Histological examination of hepatic deposits, present 6–8 weeks after orthotopic implantation, revealed well-differentiated tumours. These were shown by the use of the fluorescent dye H33342 to have a functioning network of vessels. Further immunohistochemical staining with CD31 antibody showed endothelial cell development within these liver metastases. The morphology of this secondary disease was unlike that observed after i.v. inoculation of tumour cells where there was no indication of differentiation and stromal or vascular involvement. The effects of two vaso-active agents which possess different mechanisms of action, against MAC15 tumours growing in the s.c., caecal and hepatic site, have been investigated. Both NA and HDZ showed significant effects upon tumour blood vasculature in s.c. and caecal sites although the effects of HDZ at the caecal site were not as marked as those for the s.c. tumours. These observations with orthotopic tumours suggest that vascular occlusion may be exploitable for therapeutic intervention for locally invasive colon cancers in man. HDZ and NA caused no clear vascular effect within the liver metastases which developed in the orthotopic model, although identical drug doses have been shown to be effective previously in s.c. tumours. Liver metastases were examined using the same experimental parameters as the tumours in other sites and further studies on dose scheduling and timing would be necessary to confirm that these deposits are not capable of reacting to these particular vaso-active agents.

In conclusion, the morphological appearance and vascular development of secondary disease, resulting in these animals transplanted orthotopically with colon tumours, suggest that this would be a clinically relevant model for the study of agents that act either by a vascular or an anti-angiogenic mechanism.

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