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Development of a Modified Hollow Fibre Assay for Studying Agents Targeting the Tumour Neovasculature

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Abstract. *Background: Previous studies have shown extensive vascularisation surrounding subcutaneously implanted fibres when the duration of the US National Cancer Institute (NCI) hollow fibre assay was prolonged. Materials and Methods: The feasibility of adapting the NCI assay for evaluating agents targeting the tumour vasculature was investigated in vitro and in vivo. Finally, in the optimised assay, changes in neovasculature formation around the fibres following treatment with the anti-vascular agent paclitaxel were quantified by immunohistochemistry. Results: Correlations between cell number seeded, time in culture and vascular endothelial growth factor (VEGF) secretion were seen. In vivo studies showed that transplanting single rather than 3 fibres at a site reduced inflammation, reducing the length of the fibre transplanted, as did without any significant loss in cell growth over 21 days. A statistically significant reduction in neovascularisation surrounding the fibres was seen accompanying paclitaxel treatment. Conclusion: Modifications made here to the NCI hollow fibre assay demonstrate its potential for analysing anti-tumour vasculature agents.*

Tumour neovasculature is a distinct entity from established vasculature in that it consists of a large proliferative compartment of endothelial cells, and is highly permeable. Administration of agents that can disrupt the tumour vasculature or suppress the growth of vascular endothelial cells, leading to restriction of tumour growth, is an important strategy for cancer therapy (1, 2). In recent years there has been a growth in the evaluation of novel agents which target this area and a reassessment of families of compounds already in use to look for anti-vascular properties.

In order to progress agents that target tumour vasculature into the clinic it is necessary for them to be screened in suitable preclinical assays. The main principle of these assays is that new vessel growth is stimulated in an area where there is minimal existing vasculature, or where the pattern of the existing vasculature is clearly distinguishable from the neovasculature. All currently available *in vivo* assays have their own unique features and limitations (reviewed in 3), and used on their own none is ideal. Thus, there is a requirement for the development of suitable assays for assessing tumour vasculature targeting compounds.

The hollow fibre assay was developed by the US National Cancer Institute (NCI) as a cost-effective intermediate *in vivo* screen for novel anti-cancer compounds prior to extensive evaluation in xenografts of compounds that have shown promise in its 60-cell-line *in vitro* screen (4). As used by the NCI (5, 6), the assay involves 24-hour culture *in vitro* of tumour cells inside biocompatible polyvinylidene fluoride fibres, followed by subcutaneous and intraperitoneal *(i.p.)* implantation of the fibres into mice. Compounds are administered *i.p.* for 4 days and the hollow fibres (HF) removed 2 days later and chemosensitivity assessed using a modified version of the MTT assay *in vitro* (6). Compounds proceed to xenograft evaluation if activity is seen at both subcutaneous *(s.c.)* and *i.p.* sites.

The rationale of adapting this assay for studying the effects of tumour vasculature comes from observations of the development of extensive new vascular networks surrounding *s.c.*-implanted HF loaded with murine colon adenocarcinoma cells when left *in situ* for more than the 6 days of the NCI assay (7, 8). Interestingly, this vascular network was not seen around control HF that did not

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contain any tumour cells, thus suggesting that the vascularisation was due to angiogenic factors being released by the tumour cells. As with other implant assays, such as the sponge implant assay (9), vascularity due to inflammation seen around the implantation site may interfere with the interpretation of neovascularisation.

The aims of this study were to assess modifications of the original NCI methodology in order to optimise the HF assay as a model for studying tumour neovasculature. Aspects that were covered include cell line suitability, fibre number and fibre size. As a test of the assay, the effects on neovasculature of paclitaxel were also analysed.

Materials and Methods

Cell line. The human non-small cell lung carcinoma cell line H460 was obtained from the American Tissue Type Collection (LGC Promochem, Middlesex, UK). It was routinely maintained as monolayer cultures in RPMI 1640 medium supplemented with FBS (10%), sodium pyruvate (2 mM) and L-glutamine (2 mM) (all from Sigma, UK), at 37˚C in a humidified, 5% CO_2 environment.

Hollow fibre loading. Polyvinylidene Fluoride Spectra/Por hollow fibres with an internal diameter of 1 mm and a molecular weight cut-off point of 500 kDa (Spectrum Medical Inc., Houston, TX, USA) were used. HF were cut to length, rehydrated and sterilised before cells were loaded. This involved washing the fibres in 70% ethanol for 72 hours, flushing through and immersing in distilled water, followed by autoclaving at 121˚C for 20 minutes. Fibres were then stored at 4˚C until use.

H460 cells were harvested and resuspended in RPMI 1640 medium at the required cell density. This cell preparation was then loaded into the HF, following which both ends were clamped and heat-sealed with pre-heated smooth-jawed needle holders. The HF was then cut into 1.5 cm or 2 cm lengths, which were heat sealed at both ends and transferred to 6-well plates containing sterile medium. For *in vivo* assays, incubation was for 2 hours to allow the cells to equilibrate in the fibres before transplantation, whilst for *in vitro* assays loaded HF (3 per well) were incubated for various times up to 21 days, as described below.

Characterisation of cell growth in fibres in vitro. In order to optimise the cell density, H460 cells were loaded at either 2 x 10^5 cells/ml or 1 x 10^6 cells/ml, and cell proliferation and vascular endothelial growth factor (VEGF) expression were analysed. Twenty-four hours prior to the sample point, medium was removed and fresh medium added. After 24 hours, this medium was removed and stored at –20˚C for VEGF analysis. The remaining HF were subjected to a modified MTT assay, as described previously (6), to determine the amount of viable cells in the fibres. Samples were taken over 21 days. Once all samples were collected, the amount of VEGF was determined using a commercially available ELISA kit for human VEGF (R and D Systems, Abingdon, UK) using the manufacturer's protocol. On day 21, HF were also fixed for 4 hours in 10% neutral buffered formalin, and then processed for paraffin wax embedding.

Transplantation of hollow fibres in vivo. Male NCR-Nu strain nude mice (National Cancer Institute, Bethesda, MD, USA), aged 6-8 weeks, were used. The mice were kept in cages housed in isolation cabinets in an air-conditioned room with regular alternating cycles of light and darkness. They received CRM diet (SDS, Witham, UK) and water *ad libitum*. All animal procedures were carried out under a project licence issued by the UK Home Office and UKCCCR guidelines (10) were followed throughout.

Under brief inhalation anaesthesia, a small incision was made in the left or right dorsal flank of the animal and, using a trocar, one or three HF were implanted. The mice were allowed to recover and at various times after implantation were sacrificed. Before removal of the HF, images were captured of the fibres *in situ* using a Fuji Finepix digital camera. The HF were removed and subjected to the modified MTT assay (6).

The effects of paclitaxel on the formation of neovasculature. On day 0, single HF were implanted *s.c.* as described above, either loaded with H460 cells at a density of 2.5 x 10^5 cells/ml or with cell culture

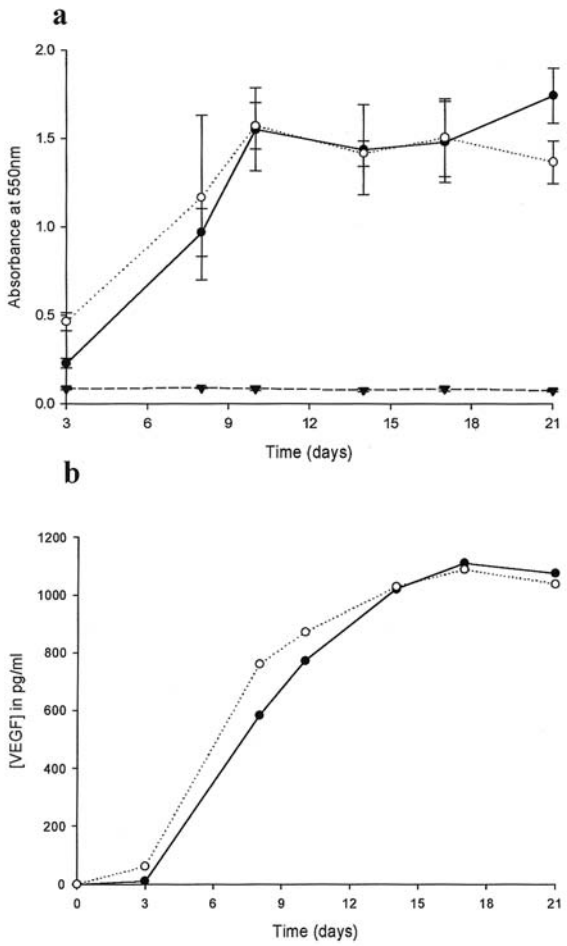

Figure 1. *Characterisation of H460 growth in HF in vitro. (a) growth curves for H460 NSCLC cells seeded at different densities. Points represent mean±S.D. (n=3)* ●, *2 x* 10^5 *cells/ml;* ○, *1 x* 10^6 *cells/ml;* ▼, *medium blank controls. (b) VEGF secretion over time for H460 cells seeded at different densities.* ●, *2 x* 10^5 *cells/ml;* ○, *1 x* 10^6 *cells/ml.*

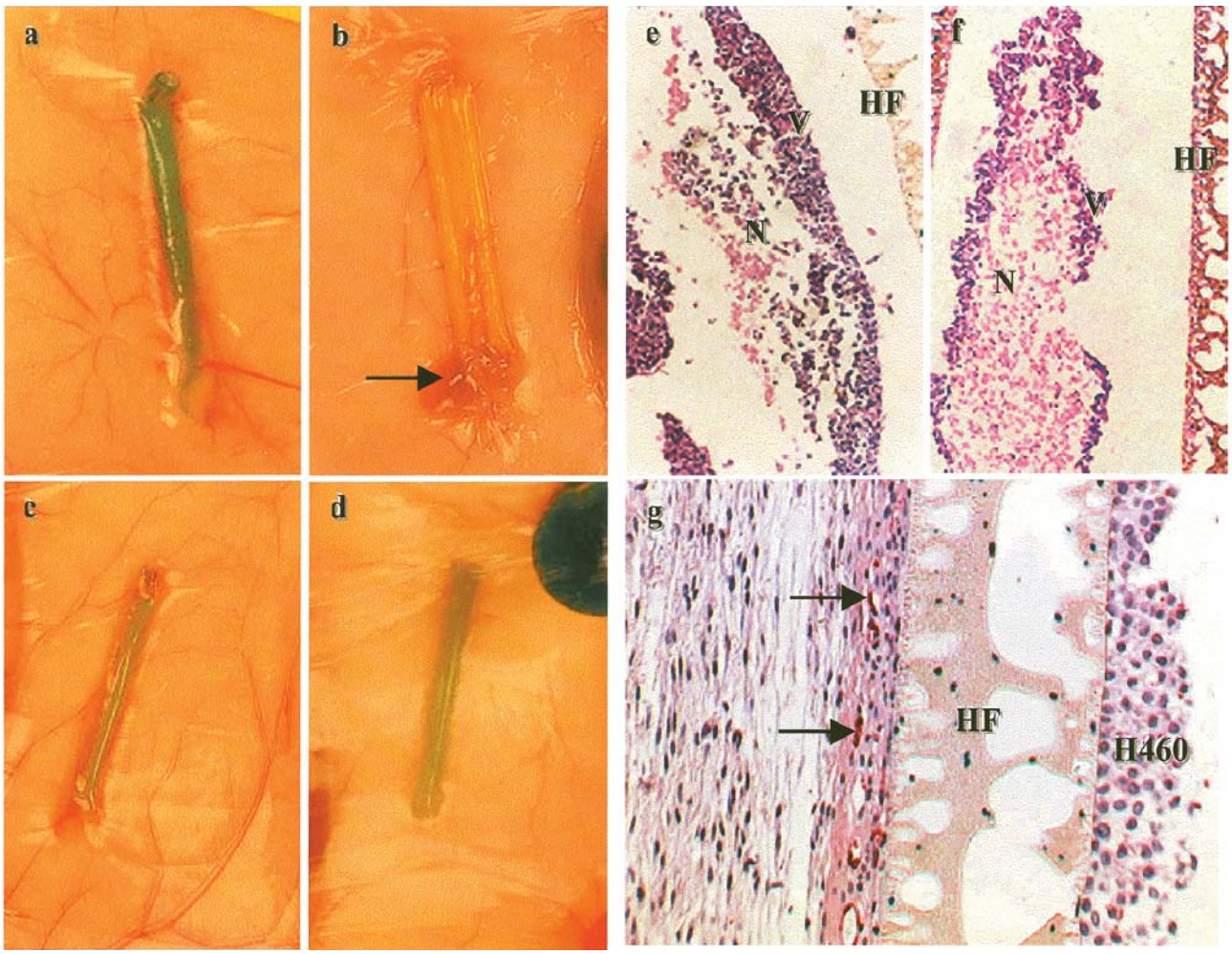

Figure 2. *(a) and (b) representative photographs of HF in situ after 21 days showing the reduction in inflammation (arrow in b) seen at the ends of fibres when one HF (a) is implanted instead of three (b). (c) and (d) representative photographs of HF in situ after 21 days showing area adjacent to fibre loaded with cells is heavily vascularised (c) compared with the area adjacent to a blank fibre (d). (e) and (f) haematoxylin and eosin-stained paraffin sections of H460 tumour cells grown in HF for 21 days showing similarities in morphology between in vitro (e) and in vivo (f) growth. Viable cell areas (V) are seen peripherally, whilst centrally necrotic areas (N) are seen. The space between the cells and the fibre wall (HF) is an artefact of processing. (g) representative photomicrograph of a paraffin section of a loaded HF in situ immunolabelled with an anti-von Willebrand Factor polyclonal antibody. Immunopositive neovascular elements (arrows) are seen adjacent to the HF. Magnifications: (a) - (d) x2; (e) and (f) x200; (g) x360.*

medium as blank controls. Five mice were implanted with HF for each experimental or control group. On days 7-11 post-transplantation, paclitaxel (Sigma, Poole, UK) dissolved in 10% DMSO: 10% cremophor EL:80% saline was administered daily as single *i.p.* injections at 20 $mgkg^{-1}$. A solvent control group received 10% DMSO: 10% cremophor EL: 80% saline under the same schedule. On day 21, the animals were euthanised and HF were excised *in situ* surrounded by a margin of skin tissue, fixed overnight in 10% neutral buffered formalin, and then processed for paraffin wax embedding and evaluation of vasculature by immuno-histochemistry, as described below.

Immunohistochemical assessment of neovasculature surrounding HF. Sections of paraffin-embedded tissue were cut with HF in longitudinal profile, de-waxed and hydrated using a graded alcohol series. The sections were then immunolabelled by standard immunohistochemical procedures using a rabbit polyclonal antibody against von Willebrand Factor (DakoCytomation, Ely, UK) with visualisation using alkaline phosphatase substrate (Vector Laboratories, Peterborough, UK). Neovasculature was quantified by counting microvessels in the granulation tissue enveloping the HF. Microvessels in the host subcutaneous and muscle tissue was excluded as these were likely to be pre-existing vessels. Images of HF *in situ* were captured digitally using a Leica DMRB microscope through a JVC 3-CCD camera and processed using AcQuis (Synoptics, Cambridge, UK) software. Statistical analysis was performed using a linear mixed effects model with the average microvessel density in the treated group compared with the untreated control group.

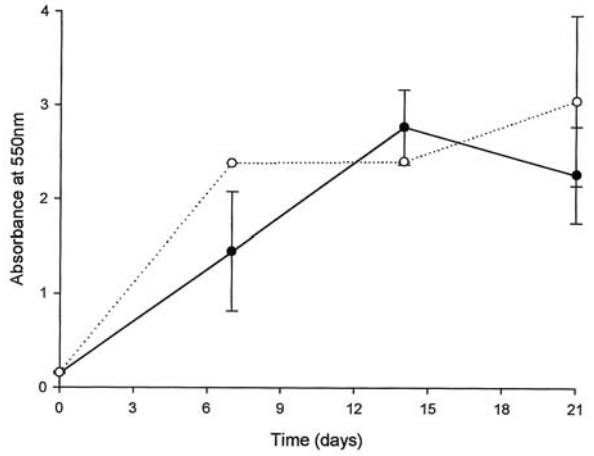

Figure 3. *Assessment of HF size on H460 growth in vivo. Points represent mean±S.D. (n=3) ●, 1.5cm HF; ○, 2.0cm HF.*

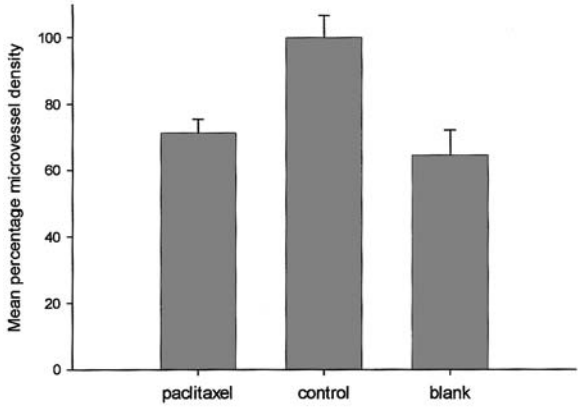

Figure 4. *The effect of treatment with paclitaxel on the neovasculature surrounding HF. Bars represent relative mean percentage microvessel density±S.D (n=5), with the density of anti-vWF-immunolabelled elements for the paclitaxel-treated and blank groups compared against the loaded control group, which was assigned a value of 100%.*

Results

Characterisation of H460 cell growth in vitro. Good growth was seen for both the cell seeding densities that were analysed (Figure 1a). After an initial exponential phase of growth for the first 10 days, the number of viable cells remained constant for the remainder of the experiment. Increased growth was initially seen for the higher seeding density, but both seeding densities peaked at a similar level, suggesting that optimum growth *in vitro* was attained. No increase in absorbance above background levels was seen for HF not loaded with cells.

In order to assess the ability of the cells to release vasculature stimulatory factors, the amount of VEGF secreted by the H460 cells was also analysed in samples taken over the time of the cell growth experiment (Figure 1b). Secretion peaked 8 days after the cells had entered the lag phase of growth. As with cell growth, after initial higher secretion seen with the higher cell seeding density, both cell concentrations peaked at a similar high level of VEGF secretion, suggesting that optimum growth in the HF has been attained.

Optimisation of in vivo HF assay. It was previously observed that considerable inflammation was seen at the HF seal when 3 HF, 2 cm in length were transplanted *s.c.* dorsally and left *in situ* for longer than a week. Thus, several modifications were assessed here to try and reduce this inflammation. By implanting one rather than 3 HF *s.c.,* the amount of inflammation seen at the ends of the HF was considerably reduced (Figure 2a and b). As another means of trying to reduce inflammation due to the presence of the HF, the length was reduced to 1.5 cm. Little difference was seen in the growth of cells in the shorter 1.5 cm HF compared with the 2.0 cm HF (Figure 3). When HF loaded with cells were observed visually they were seen to have a more developed vascular network compared with HF containing only culture medium, suggesting that the vascular formation was cell mediated (Figure 2c and d).

When the histological appearance of the growth of H460 cells within HF cultured *in vitro* and implanted *in vivo* on day 21 was analysed, it was seen that they were very similar (Figure 2e and f). For both protocols a central necrotic core of cells surrounded by a thin viable rim of cells nearer to the HF walls was observed.

The formation of neovasculature surrounding HF containing H460 cells was compared over time *versus* unloaded HF using immunohistochemical techniques (results not shown). It was seen that there was no significant difference for neovascularisation at 7 days implantation ($p=0.260$), but a significant increase in the amount of neovascularisation after 14 days implantation ($p=0.045$), which was maintained and became more evident at 21 days implantation ($p=0.003$).

The effects of paclitaxel on the formation of neovasculature. Using immunohistochemical techniques with an antibody raised against von-Willebrand Factor, novel vascular elements were seen in the tissue directly adjacent to the HF (Figure 2g). When counts were made of the immunolabelled vascular elements surrounding HF from mice treated with paclitaxel, there was a statistically significant reduction in the number of novel vascular elements ($p=0.05$) compared

with HF from control subjects. There were statistically significantly fewer novel vascular elements surrounding the unloaded HF compared to the control HF ($p<0.01$) (Figure 4), thus confirming the earlier photographic examinations of HF *in situ*.

Discussion

Previous findings that extensive vascular networks were seen to form around HF when implanted *s.c.* and left for longer than the standard 6 days of the NCI HF assay (7, 8) led us to look at ways of improving the assay, in order to adapt it for use as a preclinical screen of agents which target the tumour neovasculature. These findings showed that the neovascularisation was due to the presence of cells in the HF and, thus, angiogenic factors being released by the cells. Therefore, we characterised the cell line we were using, the H460 NSCLC line, in terms of production over time in culture in HF of one of the major promoters of neovascularisation, VEGF (11, 12). It was seen that cells secreted VEGF in a seeding-density-dependent manner. VEGF secretion has been shown to be induced by hypoxia (13), and it was seen here that peak VEGF levels were seen 4 days after the commencement of the lag phase of growth, and hence the development of hypoxic and necrotic areas in the HF. Thus, the H460 cell line is a very suitable line to use to promote neovascularisation in the HF model. Justification in applying these *in vitro* findings to the *in vivo* model comes from the fact that morphological examination of the contents of HF at 21 days showed no difference between the two systems and hence you would expect cells to be secreting VEGF in a similar manner. This VEGF secreted by the cells within the HF can diffuse out into the surrounding host tissue to exert a paracrine effect, leading to neovascularisation surrounding the HF.

When the time taken for cell-mediated angiogenesis to occur was studied, it was seen that, after 14 days implantation, there was a significant difference in the neovascularisation surrounding H460-loaded *versus* unloaded HF. This became even more evident after 21 days implantation, and thus 21 days was chosen as the optimal time for the assay. The time to see a cell-mediated effect (14 days) fits in with the *in vitro* findings. Optimal VEGF secretion was achieved around 14 days, suggesting that at this time sufficient VEGF would be available to promote angiogenesis surrounding the HF *in vivo*. This would also indicate the likelihood that neovascularisation seen at 7 days for both the loaded and unloaded HF was the result of a host inflammatory response in reaction to the initial *s.c.* implantation of HF.

Our previous experiences of implanting 3 HF together has shown that, although HF are biocompatible, areas of obvious inflammation were often seen surrounding the seals of the HF segments. Due to their formation, these are quite sharp and tend to scratch the surrounding tissue leading to inflammation. In order to try and reduce or remove this problem, we looked at decreasing the number of HF and reducing their size. Reducing the number of HF implanted at a site had a clear advantage in terms of reducing inflammation when visual observations of the HF *in situ* were carried out. This, however, removed one of the advantages of the HF assay, of being able to load different cells in the same animal, although in principle it is not so easy to distinguish neovascularisation promoted by one cell type in one HF compared with another if implanted at the same site. It is possible to implant 2 HF in the same mouse on separate flanks and obtain separate, easily distinguishable, vascular networks for both. Thus, it is still possible to keep the multicell aspect of the assay, for example being able to compare the vascularisation around a loaded and a blank HF, or cell lines with +/- angiogenic factor expression in the same mouse. Importantly, this helps to keep animal numbers used to a minimum. Another advantage of only implanting one HF at a site is that there is less chance of rupturing blood vessels surrounding the HF due to movement of the 3 HF over each other when accessing the site.

Reducing the HF size by 25% had no overall effect on the cell growth seen *in vivo*, with cell numbers peaking at the same level for both HF lengths, suggesting that the optimal sustainable load of cells has been reached. As there are negligible differences in cell growth, then the only considerations of which size HF to implant are practical ones. As the 1.5 cm HF are less restrictive to the animal's movement and thus less likely to cause inflammation around the seals due to physical trauma, we have adopted this smaller size for further work. Problems were more likely to occur from bending of the 2 cm HF when *in situ,* which can lead to exertion of pressure on the HF seals and breaks in their integrity. This was identified by analysing unloaded 2 cm HF, where absorbances above baseline were observed due to damage to the HF walls and seals which allowed influx of host cells.

One of main problems with vascular assays is the quantification of new vascular growth, and we set out to look at quantification with the HF model using an agent with well-characterised antivascular effects, paclitaxel (14). It was seen that there was a statistically significant decrease in the amount of neovascularisation adjacent to the HF as determined by immunolabelling with an antibody against von Willebrand Factor, as would be predicted. There are a couple of caveats with these findings. Firstly, there is the issue of whether the observed reduction in neovascularisation is due to a drug-induced reduction in H460 cell number in the HF, and hence reduction in the amount of VEGF being released, or is due to the paclitaxel directly affecting the endothelial cells of the neovasculature.

This could be investigated further by looking at the effects of treating with standard anti-cancer agents that have no reported effect on the tumour vasculature, or conversely with agents that specifically target the vasculature and not the tumour cells (2). Another concern is the influence of inflammation on formation of neovascular elements surrounding the HF. Although this effect can be minimised by the experimental modifications outlined here, further experiments need to be done to enhance the identification of neovasculature. One refinement could be to carry out dual immunolabelling using antibodies against inflammatory as well as neovascular markers.

This study proves the potential of the modified hollow fibre assay for analysing experimental anti-vascular agents. Promising results have been obtained with oligosaccharide derivatives of low molecular weight heparin, where reduction in vascular growth observed with the HF assay were similar to those obtained using a sponge angiogenesis assay (15).

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References

1 Folkman J: Tumour angiogenesis: therapeutic implications. New Eng J Med *285*: 1182-1186, 1971.

2 Siemann DW, Bibby MC, Dark GG, Dicker AP, Eskens FALM, Horsman MR, Marme D and LoRusso PM: Differentiation and definition of vascular-targeted therapies. Clin Cancer Res in press, 2004.

3 Hasan J, Shnyder SD, Bibby M, Double JA, Bicknel R and Jayson GC: Quantitative angiogenesis assays *in vivo* – a review. Angiogenesis *7*: 1-16, 2004.

4 Anderson LF: New drug screen assay uses fewer mice, while cutting costs. J Natl Cancer Inst *87*: 1213-1214, 1995.

5 Casciari JJ, Hollingshead MG, Alley MC, Mayo JG, Malspeis L, Miyauchi S, Grever MR and Weinstein JN: Growth and chemotherapeutic response of cells in a hollow-fibre *in vitro* solid tumour model. J Natl Cancer Inst *86*: 1846-1852, 1994.

6 Hollingshead MG, Alley MC, Camalier RF, Abbott BJ, Mayo JG, Malspeis L and Grever MR: *In vivo* cultivation of tumour cells in hollow fibres. Life Sci *57*: 131-141, 1995.

7 Phillips RM, Pearce J, Loadman PM, Bibby MC, Cooper PA, Swaine DJ and Double JA: Angiogenesis in the hollow fibre tumour model influences drug delivery to tumour cells: implications for anticancer drug screening programs. Cancer Res *58*: 5263-5266, 1998.

8 Phillips RM and Bibby MC: Hollow fibre assay for tumour angiogenesis. *In*: Methods in Molecular Medicine, vol 46: angiogenesis protocols (Murray JC, ed.). Totowa, NJ, Humana Press Inc., pp. 87-93, 2001.

9 Andrade SP, Fan T-PD and Lewis GP: Quantitative *in vivo* studies on angiogenesis in a rat sponge model. Br J Exp Pathol *68*: 755-766, 1987.

10 Workman P, Twentyman P, Balkwill F, Balmain A, Chaplin D, Double J, Embleton J, Newell D, Raymond R, Stables J, Stephens T and Wallace J: United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia. Br J Cancer *77*: 1-10, 1998.

11 Klagsbrun M and Soker S: VEGF/VPF: the angiogenesis factor found? Curr Biol *3*: 699-702, 1993.

12 Berse B, Brown LF, van de Water L, Dvorak HF and Senger DR: Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages and tumours. Mol Biol Cell *3*: 211-220, 1992.

13 Shweiki D, Itin A, Soffer D and Keshet E: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature *359*: 843-845, 1992.

14 Belotti D, Vergani V, Drudis T, Borsotti P, Pitelli MR, Viale G, Giavazzi R and Tarboletti G: The microtubule-affecting drug paclitaxel has antiangiogenic activity. Clin Cancer Res *2*: 1843-1849, 1996.

15 Hasan J, Shnyder SD, Clamp A, McGown AT, Bicknell R, Presta M, Bibby M, Stevenson K, Gallagher JT and Jayson GC: Heparin octasaccharides inhibit angiogenesis and tumour growth *in vivo*. Manuscript in preparation.

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