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## Original Paper

# Vascular Targeting of Solid and Ascites Tumours with Antibodies to Vascular Endothelial Growth Factor

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## INTRODUCTION

BECAUSE OF the exquisite specificity of their interactions with antigens, antibodies may have potential as a means of delivering cytotoxins selectively to tumours [1–5]. Many different antibody–toxin conjugates have now been tested, but, on the whole, the results have been disappointing. Even if such knotty problems as avoidance of an immune response and selection of an appropriate toxin conjugate could be resolved, several major problems would remain [6]. One such problem is that, to be effective and to avoid tumour recurrence, an antitumour antibody must recognise *all* the tumour stem cells; i.e. not only tumour cells that express differentiation antigens, but also the least differentiated stem cells whose antigenic repertoire is largely unknown. A second unresolved problem is that different antibodies need to be developed for different tumours, and possibly even for the same type of tumour growing in different patients. This is a formidable task in that more than 300 different types of tumours occur in man! However, perhaps the most difficult problem is that of antibody delivery, bringing antibodies into contact with each and every tumour cell [6, 7]. To reach tumour cells, antibodies must extravasate from blood vessels and cross tumour stroma by processes of convection and/or diffusion; they then need to penetrate tumour cell clumps that may be joined together by junctions sufficiently tight as to prevent the passage of molecules the size of antibodies. In fact, we recently demonstrated that only a small fraction of tumour cell-specific antibody that had concentrated in tumours was actually bound to tumour cells [8].

Given these difficulties, we turned to an alternate approach in which antibodies are used to target tumour blood vessels rather then tumour cells [6]. The underlying premise is that the new blood vessels that tumours induce are lined by endothelial cells expressing antigens that distinguish them from those of endothelial cells lining the microvessels of normal tissues. If this premise is correct, then making the tumour microvascular endothelium the target of antibody therapy has several important advantages as follow:

1. The newly generated blood vessels supplying many different kinds of tumours are expected to share a common set of neoantigens; therefore, antibodies developed against one or a small number of tumour vascular endothelial antigens might be effective in treating many different types of tumour, avoiding the need to develop different antibodies specific for each different type of tumour.
2. Delivery is greatly simplified in that antibodies need not cross complex tissue barriers to reach their target.
3. Tumours are particularly vulnerable to vascular damage because their blood supply is notoriously tenuous and generally lacks collaterals. Hence, antibody-toxin mediated injury to the endothelium of the blood vessels supplying tumours is expected to halt tumour blood flow and to cause the death of large numbers of tumour cells dependent on blood flow for nutrient supply.

Though attractive in principle, it has been difficult to target tumour blood vessels because of a lack of antigens unique to tumour vascular endothelium. However, recently we [9], and subsequently others [10], have identified a potential candidate protein that is selectively present in microvessels supplying many different human and animal tumours; namely, vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF). VEGF is a multifunctional cytokine, expressed and secreted at high levels by many tumour cells of animal and human origin [11–15]. VEGF is thought to have an important role in regulating tumour vessel permeability and in tumour angiogenesis, and acts by interaction with receptor tyrosine kinases, two of which have been described on vascular endothelium [16, 17]. However, VEGF is not expressed by either normal or tumour blood vessels and its detection in tumour microvascular endothelium by immunostaining is

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thought to reflect bound VEGF that had been synthesised and secreted by nearby tumour cells [9, 18–21].

These observations suggested that tumour microvessel-associated VEGF might be a useful target for antibody therapy. To test this hypothesis, we prepared affinity-purified $^{125}$I-labelled antibodies to a peptide at the VEGF N-terminus and injected these intravenously (i.v.) into mice bearing any of several syngeneic solid or ascites tumours [21–24]. Antibody distribution was compared with that of control antibodies by measurements of tumour and tissue radioactivity. Biotinylated antibodies were localised at the cellular level by avidin-peroxidase histochemistry.

## MATERIALS AND METHODS

*Antibody preparation, labelling with $^{125}$I and biotin*

Antibodies were prepared in rabbits against a peptide corresponding to the 25 amino acids comprising the N-terminus of rat VEGF except that an amide replaced the carboxyl group at the C-terminus of this peptide: APTTEGEQKAHEVVKFMDVYQRSYC [20, 22]. Rabbits were immunised at multiple intradermal sites with an emulsion containing 1 mg of rat VEGF peptide–keyhole limpet haemocyanin conjugate (Pierce Chemical Co., Rockford, Illinois, U.S.A.) in complete Freund's adjuvant; animals were subsequently boosted at 4–6 week intervals with an equivalent amount of the same conjugate in incomplete Freund's adjuvant. Collected serum was stored at −20°C prior to affinity purification on a column prepared by conjugating 5 mg of peptide to 1 g of CNBr-activated Sepharose (Pharmacia, Piscataway, New Jersey, U.S.A.). Typically, 20 ml of antiserum were passed over the column after which the column was washed thoroughly with phosphate buffered saline (PBS). Bound antibody was then eluted with 15 ml of 0.1 M acid glycine buffer, pH 2.5. Eluted antibody was immediately diluted in 2 ml of 1 M Tris buffer, pH 8.0, and dialysed against PBS. Concentrations of affinity-purified antibody were typically 0.5–1.0 mg/ml. Affinity-purified antibodies (designated Ab-VEGF-N) were prepared from multiple bleeds of three different rabbits; all shared similar properties and were highly specific for mouse VEGF as determined by immunoblotting [21].

A second anti-VEGF antibody, Ab-618, was prepared by immunising rabbits with recombinant human VEGF prepared in a baculovirus system. Preparation of this antibody and its affinity purification have been described [23]. Ab-618 bound and neutralised VEGF of both human and mouse origin.

Antibodies and several different preparations of normal rabbit IgG (nRIgG; Pierce) were radioiodinated with $^{125}$I (New England Nuclear, Boston, Massachusetts, U.S.A.) using the IODOGEN (Pierce) method [8]. Typically, 0.4 mCi of $^{125}$I was reacted with 1 mg of antibody (0.5 mg/ml). The specific activity of the iodinated product was 0.30–0.35 mCi/mg, and, after the labelled antibodies were affinity-purified, >98% of the radioactivity was precipitable by 10% (w/v) TCA (trichloracetic acid) at 4°C. Other preparations of these immunoglobulins were biotinylated for 4–6 h at pH 8.5 at room temperature using a molar ratio of 100:1 as previously described [8]. Both iodinated and biotinylated antibodies contained only negligible amounts (<3%) of aggregates as determined by HPLC-chromatography on a TSK-GEL G3000SW column (TOSO HAAS, Japan) and retained strong specific reactivity against VEGF.

*Tumour cells*

Three mouse tumours (B16 melanoma, TA3/St mammary carcinoma, and MOT ovarian tumour) were grown in solid form by injecting $2.0–2.5 \times 10^5$ cells subcutaneously (s.c.) in 5–7 week old female syngeneic mice (C57Bl/6, A/Jax and C3Heb/FeJ, respectively) [20]; tumours (5–40 mg) were harvested at 5–9 days. MOT tumours were also grown in ascites form by injecting $1 \times 10^6$ tumour cells i.p. (intraperitoneally).

In vivo *distribution studies*

Tumour-bearing mice were injected i.v. by tail vein with 10 μg $^{125}$I-labelled anti-VEGF or control antibodies in 200 μl normal saline 0.1% BSA. At various times thereafter (10 min–72 h), animals were bled from the retro-orbital space, sacrificed by $CO_2$ narcosis and exsanguinated. Tumours and a variety of normal tissues were collected, weighed and their radioactivity measured. The vascular volumes of the three tumours and normal control tissues were determined using $^{51}$Cr-radiolabelled red blood cells as previously reported [3]. Results are expressed as a percentage of injected dose (ID) per gram wet weight tissue after correction for radioactivity contributed by the blood space [3]. In some cases, excised tumours were weighed, minced, washed twice with Hanks' balanced salt solution (HBSS) at 4°C, and the radioactivity present in cell pellets and combined supernatants was measured separately to assess free and cell-bound antibody. Drinking water was supplemented with 0.1% w/v NaI beginning 24 h before injecting radiolabelled antibodies. At least 3 animals (4–6 tumours) were studied at each time point for each antibody. Analysis of variance and statistical analysis were performed using Dunnett's (parametric) or Dunn's (non-parametric) multiple comparison tests, as appropriate.

*Avidin-peroxidase staining for tissue localisation of i.v. injected biotinylated antibodies*

The distribution of biotinylated Ab-VEGF-N and control antibodies was followed in solid or ascites tumour-bearing mice. One hundred micrograms of biotinylated antibodies were injected i.v. in 200 μl 0.1% BSA-normal saline. After 24 h, solid tumour-bearing mice were killed and exsanguinated; ascites tumour-bearing mice were harvested similarly, but at 5 h because circulating antibodies were cleared rapidly from the plasma into ascites fluid. Solid tumours, peritoneal walls and mesenteries of ascites tumour-bearing mice, and various control tissues were fixed in 3.7% formaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffered saline, pH 7.6, for 1 h at room temperature and processed for avidin-biotin histochemistry [21].

## RESULTS

*Biodistribution of antibodies in mouse tumours*

Following i.v. injection, $^{125}$I-Ab-VEGF-N accumulated in all three tumours to a significantly greater extent than in a variety of normal tissues (Figures 1–3). In all three tumours, peak accumulation of Ab-VEGF-N occurred at 24 h at concentrations of 9.2–16.0% ID/g (Table 1); thereafter, Ab-

VEGF-N concentrations in tumours decreased gradually, but by less than 50% over the next two days (Figures 1–3). The lower maximum accumulation of Ab-VEGF-N in MOT compared with B16 and TA3/St tumours is probably attributable to the relatively lower vascular density of MOT tumours.

Ab-VEGF-N achieved maximal concentrations in normal tissues at earlier times ($\leqslant$ 18 h) and at substantially lower concentrations than in any of the three tumours. Thus, Ab-VEGF-N accumulation was significantly greater ($P < 0.001$) in tumours than in skeletal muscle or liver by 6 h and at all subsequent time points; however, differences between tumour and kidney or adrenal gland did not become statistically significant until 14 h and, for lung, until 18 h. At 24 h, differences in Ab-VEGF-N accumulation between any of the three tumours and all corresponding normal tissues were highly significant ($P < 0.001$); for example, at this interval the concentration of Ab-VEGF-N in B16 melanomas exceeded those in different normal tissues by a factor of 2.1 (lung) to 12.8 (skeletal muscle) (Table 1).

The tumour and normal tissue distribution of Ab-VEGF-N was compared with that of several preparations of normal rabbit IgG (nRIgG) that lacked specificity for VEGF. Both Ab-VEGF-N and nRIgG were cleared from the plasma of B16 solid tumour-bearing mice with similar kinetics (Figure 1). Also like Ab-VEGF-N, nRIgG accumulated in all three tumours to a greater extent than in skeletal muscle ($P < 0.05$ to $P < 0.01$ at different time intervals; Figures 1, 3). However, nRIgG achieved much lower peak values in all three tumours (<5% ID/g) than did Ab-VEGF-N ($P < 0.01$–$0.001$) (Table 1); also, nRIgG reached peak levels earlier, at 18 h versus 24 h for Ab-VEGF-N (Figures 1, 3). The distribution of nRIgG reached was virtually identical in the normal tissues of normal control animals and in animals bearing any of the three solid tumours (data not shown).

Finally, we sought to determine whether all antibodies to VEGF accumulated in tumours to an equivalent degree. Therefore, we labelled Ab-618 with $^{125}$I and injected it i.v. into tumour-bearing mice. To our surprise, [$^{125}$I]Ab-618 did not accumulate in TA3/St or MOT tumours to any greater extent than nRIgG (Table 1).

*Localisation of i.v. injected biotinylated antibodies in tumour microvessels*

Biotinylated Ab-VEGF-N (bAb-VEGF-N) or nRIgG (b-nRIgG) antibodies were injected i.v. into mice bearing either ascites or solid MOT tumours. Antibody distribution in peritoneal lining tissues of ascites tumour animals, in solid tumours and in two normal tissues (skeletal muscle and liver) was determined by avidin-peroxidase histochemistry.

Like solid tumours, ascites tumours elicit a striking angiogenic response [24] such that large numbers of new blood vessels develop in tissues lining the peritoneal cavity. Strong staining for i.v. injected bAb-VEGF-N antibodies was found in microvessels of the mesentery and peritoneal walls of mice bearing MOT ascites tumours (Figure 4a–d). Vessel staining was circumferential and, as determined by 1 μm Epon sections, localised primarily in the abluminal surfaces of individual endothelial cells (Figure 4b,c); this staining

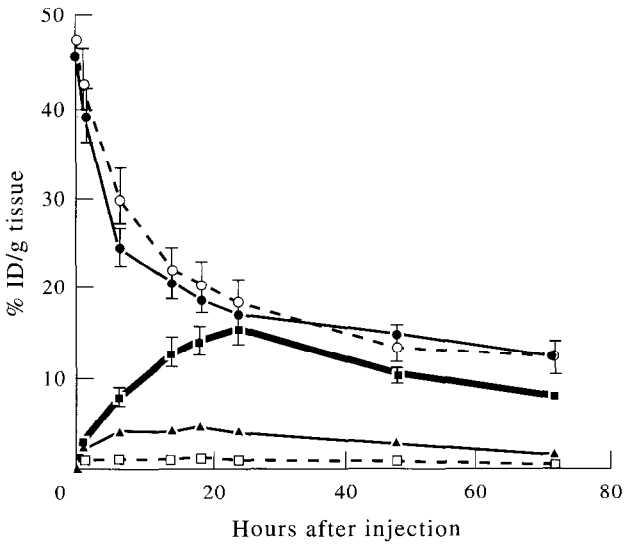


**Figure 1. Distribution of $^{125}$I-labelled Ab-VEGF-N and control normal rabbit immunoglobulin ([$^{125}$I]nRInG) in solid B16 melanomas, blood and skeletal muscle. In this and subsequent figures, data are plotted as percentage of injected dose per g of tissue (% ID/g) versus time (10 min to 72 h) after i.v. injection of 10 μg radioactive antibody or control immunoglobulin. Each data point represents the average of at least three animals (± SEM) with either one or two tumours growing in each mouse. Tumour and skeletal muscle values are corrected for radioactivity contributed by the blood space. (●—●) and (○- - -○), clearance of Ab-VEGF-N and nRIgG, respectively, from plasma; (■—■), accumulation of Ab-VPF-N in B16 melanomas; (▲—▲) and (□- - -□), accumulation of nRIgG in B16 melanomas and skeletal muscle, respectively.**

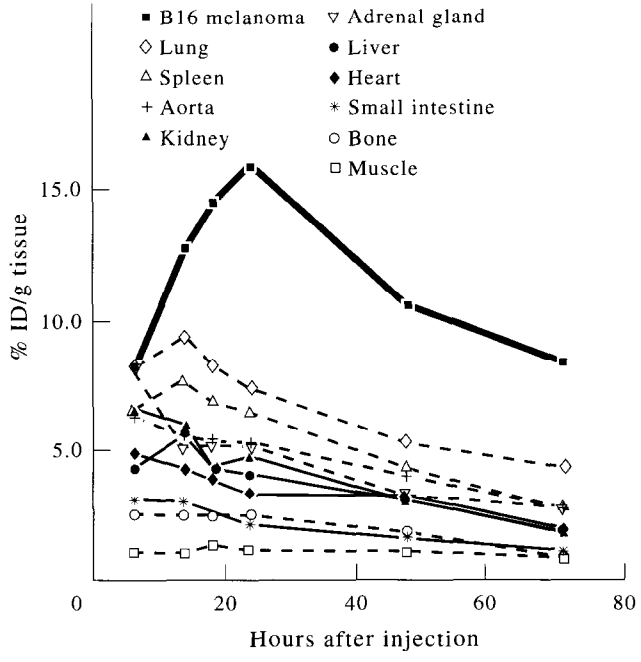


**Figure 2. Distribution of i.v. injected [$^{125}$I]Ab-VEGF-N in mice bearing syngeneic B16 melanomas.**

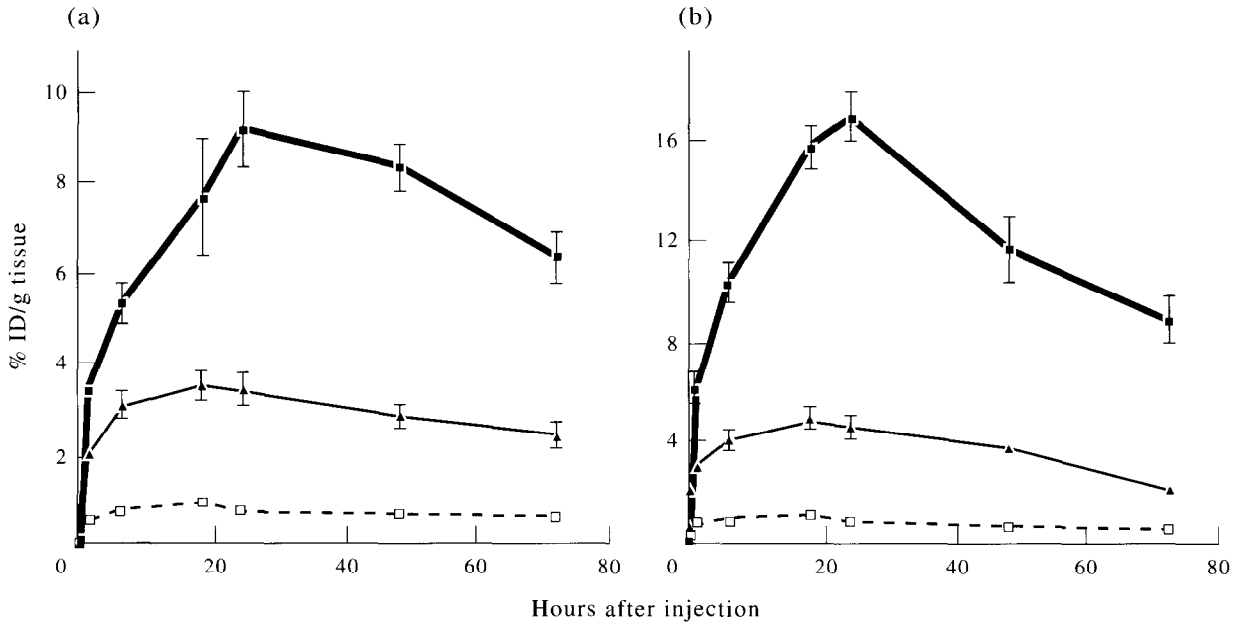


**Figure 3. Distribution of [$^{125}$I]Ab-VEGF-N or [$^{125}$I]nRIgG in syngeneic solid MOT (a) or TA3/St (b) tumours and, for comparison, of [$^{125}$I]Ab-VEGF-N in normal skeletal muscle of the same animals. [$^{125}$I]Ab-VEGF-N in MOT or TA3/St tumours (■—■); [$^{125}$I]nRIgG in MOT or TA3/St tumours (▲—▲); [$^{125}$I]Ab-VEGF-N in skeletal muscle (□- - -□).**

pattern is similar to that demonstrated previously in immunohistochemical experiments when antibodies were applied to tumour sections and localised, at either the light or electron microscopic levels [9, 18–21]. No staining was observed in vessels at a distance from the tumour–host interface greater than approximately 0.3 mm. Similar intense staining was observed in microvessels supplying MOT solid tumours (Figure 4f,g); in addition, tumour stromal matrix immediately surrounding stained vessels was also sometimes stained (Figure 4f). In contrast, no vessel staining was observed in association with either ascites or solid MOT tumours in mice injected i.v. with b-nRIgG (Figure 4e,h), although faint stromal staining was sometimes observed focally (Figure 4h).

## DISCUSSION

The data reported here indicate that Ab-VEGF-N directed against the VEGF N-terminus accumulated selectively in solid and ascites tumours and, more specifically, in tumour microvascular endothelium. Intravenously administered, affinity-purified Ab-VEGF-N antibodies achieved maximum concentrations in three syngeneic mouse tumours of 9–16% ID/gram. These concentrations greatly exceeded those measured in normal tissues and approached concentrations reported in the literature for antibodies directed against tumour cell surface antigens [3]. Such levels are impressive in that tumour cells greatly outnumber vascular endothelial cells and thus would be expected to provide a much larger antigen pool for antibody binding.

Accumulation of Ab-VEGF-N was maximal in all three solid tumours 24 h after i.v. injection, at which time tumour concentrations exceeded concentrations in the various normal tissues studied by factors of more than 2–13-fold. Previous immunohistochemical studies performed on fixed and processed sections of these and other tumours have demonstrated that the Ab-VEGF-N stained VEGF that was bound to tumour microvessels and particularly the abluminal surface of tumour microvascular endothelium [9, 18–21]. The present experiments have now extended these findings, demonstrating that these antibodies, when biotinylated and

*Table* 1. *Accumulation of Ab-VEGF-N, Ab-*618 *and nRIgG in solid mouse tumours* 24 *h after i.v. injection of* 10 *μg of* $^{125}$I-*labelled protein. Data are expressed as % ID/g tumour. Each datum represents the mean ± SE of* 4 *to* 6 *separate tumours*

| Antibodies | Tumours | | | Skeletal muscle |
|---|---|---|---|---|
| | B16 | TA3/St | MOT | |
| Ab-VEGF-N | 15.4 ± 1.3 | 16.0 ± 1.0 | 9.2 ± 0.7 | 1.2 |
| nRIgG | 4.3 ± 0.3 | 4.7 ± 0.5 | 3.4 ± 0.4 | 1.2 |
| Ab-618 | — | 2.9 ± 0.1 | 2.5 ± 0.2 | — |
| Ratios | | | | |
| Ab-VEGF-N: tumour/muscle | 12.8 | 13.3 | 7.7 | — |
| Tumour: Ab-VEGF-N/nRIgG | 3.6 | 3.4 | 2.7 | 1.0 |
| Tumour: Ab-VEGF-N/Ab-618 | — | 5.5 | 3.7 | — |

Accumulation of [$^{125}$I]Ab-VEGF-N in all three mouse tumours was significantly greater than that of either [$^{125}$I]nRIgG or [$^{125}$I]Ab-618 ($P < 0.001 - < 0.0001$).

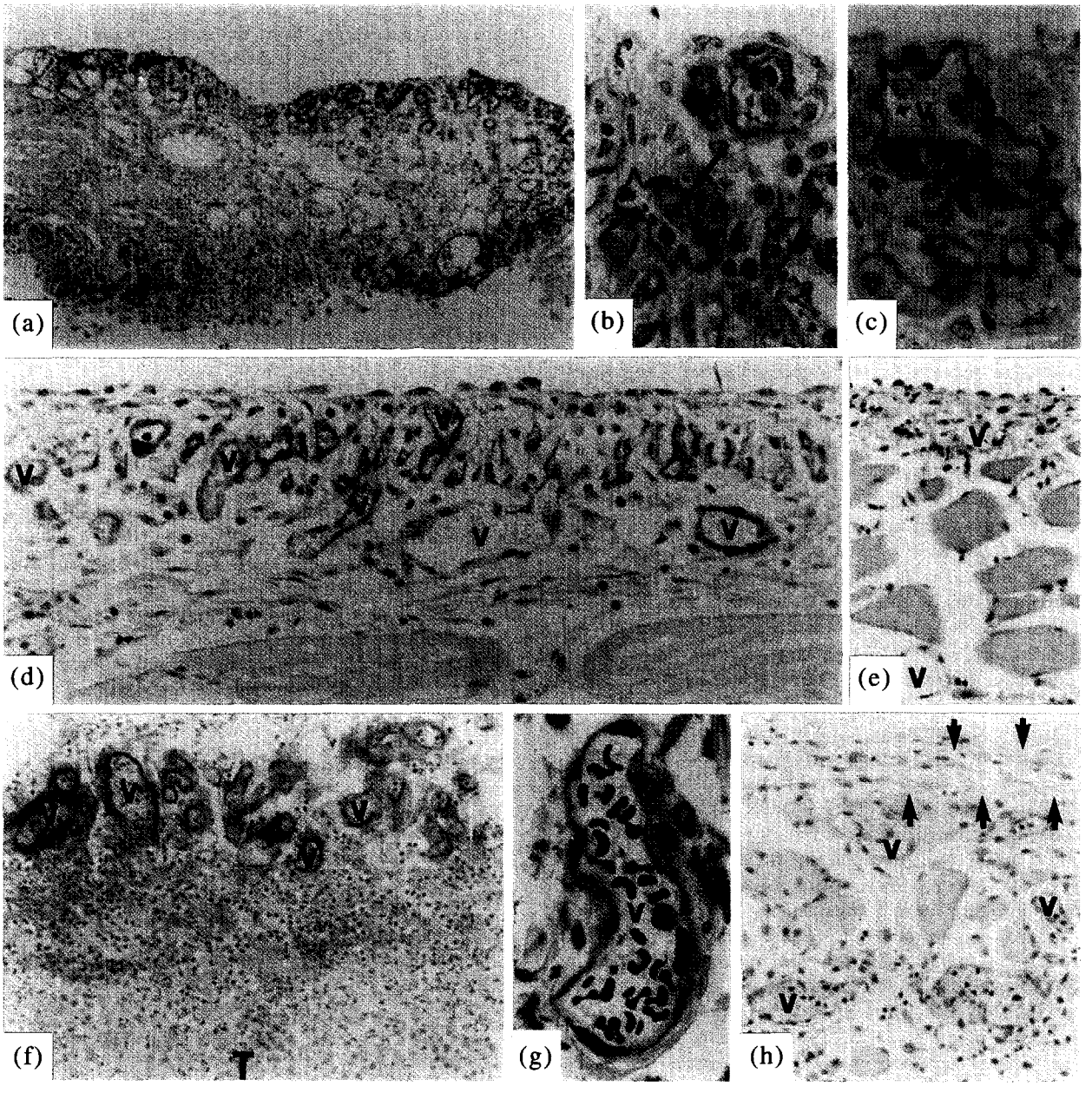


**Figure 4. Avidin-peroxidase staining of syngeneic MOT solid (5 day) or ascites (14 day) tumours harvested 24 h after i.v. injection of 100 μg of bAb-VEGF-N or bnRIgG. All figures represent photomicrographs of either 4 μm frozen sections (a, d, e, h) or 1 μm plastic sections (b, c, g) stained with avidin-peroxidase and counterstained with either Mayer's haematoxylin or toluidine blue. (a) Low-power overview showing intense staining of new, tumour-induced microvessels in mesentery of ascites tumour-bearing animal; (b, c) mesenteric microvessels at higher magnification, demonstrating largely abluminal pattern of endothelial cell staining; (d) low-power overview showing intense staining of new, tumour-induced microvessels in peritoneal wall of ascites tumour-bearing mouse; (e) corresponding photomicrograph of peritoneal wall microvessels of MOT ascites tumour-bearing mouse injected i.v. with bnRIgG. Note lack of detectable staining; (f) avidin-peroxidase staining of MOT solid tumour in a mouse injected i.v. with bAb-VEGF-N. Note intense staining of vessels and weaker staining of matrix at tumour–host interface; (g) higher magnification view of a labelled vessel in a MOT solid tumour; (h) solid MOT tumour growing in a mouse injected i.v. with bnRIgG. Vessels are negative, but matrix is faintly stained (arrows). v, vessels; T, tumour. Magnifications: (a) 94×; (b) 508×; (c) 660×; (d) 278×; (e, f) 168×; (g) 857×; (h) 210×.**

administered i.v. have access to tumour microvascular endothelium-associated VEGF in living animals. Moreover, the concentrations of Ab-VEGF-N achieved in the three different solid tumours we studied were proportional to overall tumour vascularity: B16 = TA3/St > MOT [24] (unpublished data). These findings, therefore, demonstrate the feasibility of using antibodies directed against the N-terminus of VEGF as a delivery vehicle for selectively targeting the microvasculature of solid and ascites tumours. bAb-VEGF-N also concentrated in microvessels lining the peritoneal cavities of ascites tumour-bearing mice where VEGF has also been demonstrated previously by immunohistochemistry [20].

In contrast to our results with Ab-VEGF-N that is directed against the VEGF-N-terminus, a polyclonal antibody raised against whole recombinant VEGF protein (Ab-618) did not accumulate selectively in tumours and was distributed *in vivo* like normal rabbit IgG. This result, repeated several times, was surprising in that Ab-618 immunoprecipitated both mouse and human VEGF from solution and bound VEGF in Western blots more effectively than Ab-VEGF-N; Ab-618 was also more effective than Ab-VEGF-N in neutralising VEGF's biological activities. We suggest that these experiments indicate that once VEGF has bound to microvessels, many of its epitopes are no longer accessible to an antibody; it is perhaps fortuitous that epitopes associated with the VEGF N-terminus retained their capacity to bind specific antibodies after becoming associated with tumour microvascular endothelium. We also conclude that the pool of free VEGF present in tumours is relatively small and insufficient to concentrate Ab-618 in amounts that significantly affect its overall tissue distribution as measured by the failure of $^{125}$I-Ab-618 to accumulate in tumours in concentrations significantly different from that of $^{125}$I-nRIgG.

Others have reported that systemic administration of neutralising antibodies directed against VEGF inhibit the growth of solid tumours [25, 26]. The antibodies used in such experiments had strong VEGF neutralising properties, but were not reported to react with VEGF deposited in tumour vascular endothelium, and their pharmacokinetics were not investigated. These antibodies are thought to act by intercepting and neutralising tumour-secreted VEGF prior to its interaction with microvascular endothelium, thereby inhibiting tumour angiogenesis. Antibody treatment was started at the time of tumour cell injection when tumour cell numbers were low and prior to the onset of tumour vascularisation. These conditions are unlikely to be achievable in the case of primary human tumours, although the approach might be effective in preventing the growth of freshly seeded tumour metastases. In contrast, we have adopted a complementary therapeutic strategy; by using Ab-VEGF-N as a vehicle with the potential of delivering toxins to existing tumour microvascular endothelium, we hope to destroy pre-existing tumour microvasculature and thereby kill large masses of tumour cells.

It is well known that tumour microvessels are distinctly hyperpermeable in comparison with the vessels of normal tissues, a property that is thought to depend on their response to tumour cell-secreted VEGF [12, 27, 28]. As a consequence of this hyperpermeability, circulating proteins extravasate from tumour vessels at increased rates compared with normal vessels and, other properties being equal, it is to be expected that tumour-specific and non-specific antibodies extravasate from tumour vessels at similar rates. The hyperpermeability of tumour microvessels accounts for the fact that non-specific antibodies, such as normal rabbit IgG, enter tumours much more readily than into most normal tissues [3]. Thus, although tumour concentrations of Ab-VEGF-N were $\geqslant$ 3-fold higher than those of several batches of normal rabbit IgG and Ab-618, it remains true that normal rabbit IgG and Ab-618 also accumulated in tumours in amounts exceeding those of normal tissues (Figures 1 and 3; Table 1). Taken together, these data indicate that the substantially greater accumulation of Ab-VEGF-N than normal IgG in tumours reflects reduced efflux of these antibodies from tumours, attributable to binding of specific antibody to tumour blood vessel-associated antigen. It is very likely that such success as has been achieved by a number of investigators in localising systemically administered monoclonal antibodies (and even such non-antibody proteins as fibrinogen) to tumours is attributable to tumour vessel hyperpermeability [29, 30].

Taken together, our data indicate that Ab-VEGF-N offers a promising vehicle for targeting tumour microvessels. The rationale behind this approach is that such antibodies can be used to ferry cytotoxins selectively to tumour microvessels where they can act to ablate the tumour microvasculature, thereby affecting the destruction of large masses of tumour cells which are dependent on an adequate vascular supply for their nutritional requirements. There are many reasons why this approach is advantageous compared with the use of antibodies with specificity for tumour cells; e.g. microvessels are more accessible to antibodies than tumour cells, antibodies directed against endothelium-associated VEGF should be effective in many different kinds of tumour. Perhaps the most compelling of these arguments is that halting the flow of a single tumour microvessel will have a multiplier effect resulting in the death of all of the tumour cells dependent on that vessel.

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