IMMUNOHISTOCHEMICAL ANALYSIS OF INTEGRIN αVβ3 EXPRESSION ON TUMOR-ASSOCIATED VESSELS OF HUMAN CARCINOMAS

Regina MAX1,2, Roland R.C.M. GERRITSEN1, Peet T.G.A. NOOIJEN1, Simon L. GOODMAN3, Arne SUTTER3, Ulrich KEILHOLZ3, Dirk J. RUITTER1 and Robert M.W. DE WAAL*1

1Department of Pathology, University Hospital Nijmegen, The Netherlands
2Department of Internal Medicine V, University Hospital Heidelberg, Germany
3Merck KGaA, Pre-Clinical Pharmaceutical Research (IMO), Darmstadt, Germany

Expression of the αvβ3 integrin is upregulated on sprouting endothelia. Systemic application of antibody or peptidic inhibitors of αvβ3 function disrupts tumor angiogenesis and reduces growth and invasiveness of human tumors in animal models. We systematically investigated αvβ3 expression on tumor-associated vessels of 4 different human epithelial tumors and the corresponding normal tissues by means of immunohistochemistry on frozen sections using the αvβ3-specific monoclonal antibody LM609. Variable levels of LM609 staining were found in all carcinoma lesions. A considerable number of tumor tissues (35/50) expressed αvβ3 on more than 50% of their vessels. Inflammatory infiltrates and the possibly hypoxic conditions near necrotic areas of tumors were accompanied by an increased αvβ3 expression. Remarkably, the vasculature in apparently normal tissue also stained for αvβ3. However, the percentages of stained vessels and their staining intensity were lower than in neoplastic tissues. Besides the vascular αvβ3 expression, several extravascular cell types stained positive, in both normal and tumor specimens. Taken together, our findings show a considerable number of colon, pancreas, lung and breast carcinoma lesions with many αvβ3-expressing vessels that could be targets for anti-αvβ3-therapy. Int. J. Cancer 71:320–324, 1997.

© 1997 Wiley-Liss, Inc.

Angiogenesis, the formation of new blood vessels from pre-existing ones, plays a key role in development, wound repair and inflammation. This process also contributes to pathological conditions such as diabetic retinopathy, rheumatoid arthritis and cancer. After solid tumors reach a volume of 1–2 mm³, their further outgrowth is strictly dependent on angiogenesis (Folkman, 1992). In several human tumors an association between vascular density—quantified by microvessel counting—and tumor metastasis or outgrowth is strictly dependent on angiogenesis (Folkman, 1992). Several studies have investigated the distribution of αv- and β3-integrins on vessels (Mechtersheimer et al., 1994; Luscinakas et al., 1994), but until now the use of a complex-specific antibody against the most interesting target, αvβ3, has been limited. As different αv- and β3-integrins are known, the reagents directed to individual chains must give ambiguous results.

Here, our interest was to investigate which solid human tumors show marked αvβ3 expression on their vascular beds and could thus possibly be targets for treatment with αvβ3-antagonists. We compared the αvβ3 expression on the vasculature of human tumors with that in the corresponding normal tissues by means of immunohistochemical analysis of serial frozen sections.

MATERIAL AND METHODS

Clinical material

Frozen surgical material and histologically normal specimens from autopsy, performed within 4 hr post-mortem (Department of Pathology, University Hospital Nijmegen) were used. The following samples were investigated: colorectal adenocarcinomas (n = 15), pancreas carcinomas (n = 10), lung carcinomas (n = 13), breast carcinomas (n = 12) and normal colon (n = 5), pancreatic (n = 5), lung (n = 3) and breast tissue (n = 3).

Immunohistochemical techniques

Sections of the frozen material were stained with the anti-αvβ3 monoclonal antibody LM609 (a kind gift of Dr. D. Cheresh, La Jolla, CA) and the anti-endothelial cell MAb PAL-E, a specific marker for small veins, venules and capillaries, which was used as a reference for endothelial staining (Schlingemann et al., 1985). Serial 4-μm sections of frozen tissue were air-dried overnight at room temperature (RT), fixed in acetone for 10 min, air-dried and then incubated with PAL-E (undiluted hybridoma supernatant) and LM609 (2.55 μg/ml) at RT for 60 min. Overnight drying did not result in artifactual or background staining with either antibody. After the sections had been rinsed in PBS, PAL-E was detected by incubation at RT with a alkaline phosphatase(AP)-conjugated rabbit anti-mouse antibody (RAM-AP, Dakopatts, Glostrup, Denmark). For LM609, in addition to RAM-AP, a third step with AP-conjugated swine anti-rabbit antibody (Dakopatts) was necessary to enhance the specific staining signal. All MAbs were diluted in PBS (pH = 7.4) containing 1% BSA. As substrates, Naphthol AS-TR phosphate in TRIS-buffer, levamisole and Fast red TR were used.

Recent findings in a severe combined immunodeficiency (SCID) mouse/human skin chimeric model indicated that the intravenous application of a function-blocking monoclonal antibody (MAb) (LM609) directed to the αvβ3-complex is able to reduce the growth and invasiveness of human tumors. This seems to be due to the perturbation of angiogenesis induced by the tumors, with no apparent effect on surrounding normal tissue (Brooks et al., 1995). In a chorioallantoic membrane (CAM) model, both peptidic and antibody antagonists of αvβ3 strongly blocked tumor-induced angiogenesis (Brooks et al., 1994a,b). These data suggest that integrin αvβ3 might be a useful therapeutic target for cancer and other diseases characterized by pathological angiogenesis.

Several studies have investigated the distribution of αv- and β3-integrins on vessels (Mechtersheimer et al., 1994; Luscinakas et al., 1994), but until now the use of a complex-specific antibody against the most interesting target, αvβ3, has been limited. As different αv- and β3-integrins are known, the reagents directed to individual chains must give ambiguous results.

Here, our interest was to investigate which solid human tumors show marked αvβ3 expression on their vascular beds and could thus possibly be targets for treatment with αvβ3-antagonists. We compared the αvβ3 expression on the vasculature of human tumors with that in the corresponding normal tissues by means of immunohistochemical analysis of serial frozen sections.

MATERIAL AND METHODS

Clinical material

Frozen surgical material and histologically normal specimens from autopsy, performed within 4 hr post-mortem (Department of Pathology, University Hospital Nijmegen) were used. The following samples were investigated: colorectal adenocarcinomas (n = 15), pancreas carcinomas (n = 10), lung carcinomas (n = 13), breast carcinomas (n = 12) and normal colon (n = 5), pancreatic (n = 5), lung (n = 3) and breast tissue (n = 3).

Immunohistochemical techniques

Sections of the frozen material were stained with the anti-αvβ3 monoclonal antibody LM609 (a kind gift of Dr. D. Cheresh, La Jolla, CA) and the anti-endothelial cell MAb PAL-E, a specific marker for small veins, venules and capillaries, which was used as a reference for endothelial staining (Schlingemann et al., 1985). Serial 4-μm sections of frozen tissue were air-dried overnight at room temperature (RT), fixed in acetone for 10 min, again air-dried and then incubated with PAL-E (undiluted hybridoma supernatant) and LM609 (2.55 μg/ml) at RT for 60 min. Overnight drying did not result in artifactual or background staining with either antibody. After the sections had been rinsed in PBS, PAL-E was detected by incubation at RT with a alkaline phosphatase(AP)-conjugated rabbit anti-mouse antibody (RAM-AP, Dakopatts, Glostrup, Denmark). For LM609, in addition to RAM-AP, a third step with AP-conjugated swine anti-rabbit antibody (Dakopatts) was necessary to enhance the specific staining signal. All MAbs were diluted in PBS (pH = 7.4) containing 1% BSA. As substrates, Naphthol AS-TR phosphate in TRIS-buffer, levamisole and Fast red TR were used.
VASCULAR avβ3 EXPRESSION IN HUMAN CARCINOMAS

**Figure 1** - Microphotographs of normal colonic tissue. Serial cryostat sections were stained with the endothelial marker PAL-E (a) and LM609 anti-avβ3 MAb (b). Arrowheads indicate an arteriole, arrows indicate a venule. M, Lamina mucosa; S, tela submucosa; m, tunica muscularis. Bars: 66 μm.

Finally, sections were counterstained with Mayer's hematoxylin and mounted with Kaiser's glycerol-gelatin (Merck, Darmstadt, Germany). This protocol was used for all lesions except the colonic tissues. As the endogenous alkaline phosphatase in colonic tissue causes non-specific background staining, second and third antibodies were replaced. For PAL-E a peroxidase-conjugated rabbit anti-mouse antibody (RAMPO, Dakopatts) was used, for LM609 the peroxidase-based Vectastain elite ABC kit (Vector, Burlingame, CA). This was followed by incubation with 0.5 mg/ml DAB (3,3' diaminobenzidine-tetrahydrochloride, Sigma (St. Louis, MO)). Then the sections were treated for 5 min with a 0.9% NaCl solution containing 0.5% CuSO4 and counterstained with Mayer's hematoxylin. Finally, the sections were dehydrated and mounted with Permount (BooM, Meppel, The Netherlands). Controls consisted of incubations replacing the primary antibodies with PBS/BSA, with other anti-integrin MAbs of the same isotype but with different specificity, and with an unrelated Mab of the IgG1 isotype.

**Vascular avβ3 expression**

Stained slides were scored independently for antigen expression by 2 investigators using light microscopy. On sections stained with the endothelial marker PAL-E, any red- (AP) or brown-staining (DAB) cell or cluster of cells was considered to be a single countable microvessel independent of the presence of a lumen. Individual microvessel counts were made in at least 4 different fields per section (magnification ×250) and compared with similar areas of serial sections stained for avβ3. Each PAL-E-defined (micro-)vessel showing LM609 staining above the parallel second-layer controls was counted as positive. A magnification of ×250 was necessary to unequivocally distinguish staining of endothelial cells from that of media.

Results are expressed as mean percentage of avβ3-expressing (micro-)vessels as a fraction of the PAL-E-defined (micro-)vessels.

**RESULTS**

Table I shows the mean number, standard deviation and range of evaluated PAL-E-positive structures per field in neoplastic and normal tissues. The percentages of avβ3-expressing (micro-)vessels are summarized in Table II. To verify the specificity of the

---

**Table I - Mean number of evaluated PAL-E-defined vessels per field (magnification ×250) in neoplastic and normal tissues**

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Number of evaluated vessels per field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon carcinoma n = 15</td>
<td>31.3 ± 13.2 14-66</td>
</tr>
<tr>
<td>Normal colon n = 5</td>
<td>17.8 ± 1.3 16-20</td>
</tr>
<tr>
<td>Pancreas carcinoma n = 10</td>
<td>54.2 ± 24.2 12-89</td>
</tr>
<tr>
<td>Normal pancreas n = 5</td>
<td>142.2 ± 8.7 130-157</td>
</tr>
<tr>
<td>Lung carcinoma n = 13</td>
<td>44.7 ± 25 7-95</td>
</tr>
<tr>
<td>Normal lung n = 3</td>
<td>83 ± 31 54-126</td>
</tr>
<tr>
<td>Breast carcinoma n = 12</td>
<td>46.8 ± 16.8 15-80</td>
</tr>
<tr>
<td>Normal breast n = 3</td>
<td>9.3 ± 2.1 7-12</td>
</tr>
</tbody>
</table>

1SD, standard deviation.

This study was not performed to compare vessel densities of normal and cancerous tissues and the numbers in this table should not be taken as statistically validated data of vessel density in the different tissues.

---

**Table II - Mean percentage of avβ3-positive vessels out of four different fields/section. Each □ stands for one normal tissue, each ■ stands for one carcinoma.**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Estimated proportion of stained microvessels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-30%</td>
</tr>
<tr>
<td>Colon carcinoma n = 15</td>
<td>■■■■</td>
</tr>
<tr>
<td>Normal colon n = 5</td>
<td>□</td>
</tr>
<tr>
<td>Pancreas carcinoma n = 10</td>
<td>■</td>
</tr>
<tr>
<td>Normal pancreas n = 5</td>
<td>□</td>
</tr>
<tr>
<td>Lung carcinoma n = 13</td>
<td>■■■■</td>
</tr>
<tr>
<td>Normal lung n = 3</td>
<td>□</td>
</tr>
<tr>
<td>Breast carcinoma n = 12</td>
<td>■■■■</td>
</tr>
<tr>
<td>Normal breast n = 3</td>
<td>□</td>
</tr>
</tbody>
</table>

- This study was not performed to compare vessel densities of normal and cancerous tissues and the numbers in this table should not be taken as statistically validated data of vessel density in the different tissues.
αβ3 stainings, a comparison was made with the stainings of 2 different antibodies directed to the αβ3-complex. Both anti-αβ3 antibodies showed a characteristically different staining pattern from that obtained with LM609 (not shown).

In all carcinoma lesions investigated, αβ3-expressing vessels were found. The percentages of LM609-positive vessels as a fraction of the entire actual vasculature, defined by PAL-E-staining, showed wide variations, and all tumor types had examples of single lesions with less than 50% αβ3-expressing vessels. Some of these lesions, showing LM609-staining on endothelial as well as on other cells, were difficult to evaluate as the αβ3 expression of tumor and/or stromal cells partly obscured the vascular staining. Therefore, the degree of vascular expression may have been underestimated in these sections. However, 35 out of 50 carcinoma lesions of different origins showed αβ3 expression on more than half of their tumor-associated vessels.

Colon

Normal colonic tissue showed αβ3 expression on different mesenchymal cell types. The tunica muscularis consisted of strongly stained smooth-muscle cells, making it impossible to see vascular staining. In the lamina mucosa the same feature appeared. Staining of connective-tissue cells partially obscured the vascular αβ3 expression, but single capillaries showing LM609-staining on endothelial as well as on other cells, were difficult to evaluate as the αβ3 expression of tumor and/or stromal cells partly obscured the vascular staining. Therefore, the degree of vascular expression may have been underestimated in these sections. However, 35 out of 50 carcinoma lesions of different origins showed αβ3 expression on more than half of their tumor-associated vessels.

Pancreas

In normal pancreatic tissue the percentage of αβ3-expressing vessels ranged from 25% to 60%. The lesions of pancreatic carcinomas showed 30% to 80% LM609-positive vessels. In both lesions with less than 50% αβ3-expressing vessels, strongly positive stromal and tumor cells were seen in about half of the section, partially obscuring the LM609 staining. In 2 carcinoma lesions with more than 70% αβ3-expressing vessels, normal pancreas tissue could be found in the same section. The percentage of vessels stained with LM609 in such areas was lower (40% to 60%) than in the carcinoma and the staining intensity on individual vessels was weaker (Fig. 2a–d). Single lesions showed inflammatory infiltrates surrounded by many αβ3-expressing vessels.

Lung

The percentage of LM609-positive vessels in normal lung tissues was less than 35% in all lesions investigated. Only a few capillaries within the interalveolar septa were stained by LM609, whereas all detectable small and medium-sized venules and arterioles expressed αβ3. The arterioles especially showed staining not only of endothelial, but also of smooth-muscle cells in the tunica media. Out of 13 lung carcinomas, 9 showed αβ3 expression on more than 50% of their vessels. The 2 lesions with the highest percentages of stained vessels (80% and 90%) were partly necrotizing, squamous-cell carcinomas. In some lesions extensive inflammatory infiltrates could be seen. Within and surrounding these infiltrates the percentage of αβ3-expressing vessels was high and the staining intensity was strong. Stromal cells were stained with LM609 in some cases and a few lesions showed positively stained tumor cells.

Breast

Only a weak LM609 staining of less than 25% of the PAL-E-defined vessels was detectable in normal breast tissues. The percentage of αβ3-expressing vessels in breast carcinomas ranged from 40% to 70%. One lesion showed normal breast and carcinoma tissue within the same section. The vasculature in the tumorous part showed a higher expression of αβ3 (both percentage and staining intensity). In single carcinoma tissues, inflammatory infiltrates surrounded by many αβ3-expressing vessels could be seen. Most lesions showed LM609 staining of stromal cells, while a few showed positive tumor cells. In some carcinomas, αβ3 was expressed on a cell layer surrounding tumor-cell nests. Schwann cells also expressed αβ3.

In the evaluation of αβ3 expression in normal tissue, we had to consider that we used autopsy tissues which had already been subjected to hypoxic conditions. Since integrin expression might have been altered by post mortem hypoxia, we used rabbit tissue to investigate whether the time between death and tissue sampling influenced vascular αβ3 expression. LM609 recognizes αβ3 on angiogenic vessels in rabbits (Friedlander et al., 1995). On the vasculature of normal rabbit tissues, little αβ3 expression could be detected, irrespective of the time which had elapsed between death of the animal and tissue sampling.

DISCUSSION

Integrin αβ3 is strongly implicated in tumor angiogenesis (Brooks et al., 1994a,b; 1995). We investigated 4 different human carcinomas and the corresponding normal tissues to identify which expressed αβ3 on vessels. By contrast with recent studies with antibodies directed to individual integrin chains (Mechtersheimer et al., 1994; Luscinskas and Lawler, 1994), we used LM609 that binds only to intact and functionally active αβ3-complex (Cheresh and Sprio, 1987), and used PAL-E (Schlingemann et al., 1985) rather than anti-factor-VIII-related antigen antibody as an endothelial marker. This allowed us to routinely distinguish staining of endothelial cells and smooth-muscle cells of the media. In addition to the study of αβ3 staining detailed here, we examined the distribution of the αβ5 integrin to investigate whether the observed αβ3 distribution was specific. αβ5-complex specific antibodies gave staining patterns which were characteristically different from those of αβ3, showing, in particular, a much broader tissue distribution and thereby suggesting the specificity of the immunohistochemical staining for αβ3.

αβ3 is expressed on angiogenic vessels in man, chicken and rabbit (Brooks et al., 1994a; Friedlander et al., 1995) and expression and ligation of αβ3 on endothelial cells of sprouting vessels seems to protect them from apoptosis (Brooks et al., 1994b). This led to the hypothesis that αβ3 expression is part of the specific phenotype of angiogenic vessels (Brooks et al., 1994b).

Here, we observed extensive αβ3 expression on vessels in normal tissues. In most organs αβ3 was expressed on less than 50% of the PAL-E-defined vessels, but 1 out of 5 pancreatic and 4 out of 5 colonic tissues showed staining of more than 50% of their vasculature. In all lesions, small and medium-sized vessels expressed αβ3. LM609 stained not only the endothelia but also the smooth-muscle cells in the vessel media. The distribution of αβ3 observed by us in normal tissues is similar to that previously reported for isolated αv- and β3-subchains (Mechtersheimer et al., 1994). The interpretation of those earlier studies, however, was hampered by the fact that they did not use complex-specific antibodies and both αv and β3 potentially combine with multiple partner chains. We were able to show that αβ3 expression on normal vasculature is probably not a post mortem artefact since no significant differences in LM609 could be found on vessels of any of the rabbit organs studied, comparing samples taken from these immediately, 1 hr or 8 hr after death. To confirm this on human tissue, we are now investigating different colorectal and dermal biopsies.

Comparison of the αβ3 expression in neoplastic and normal tissue of pancreas, lung and breast shows an increase in LM609 staining in many carcinomas. This supports a link between angiogenesis and enhanced αβ3 expression (Brooks et al., 1995).
Besides the differences in the numbers of αvβ3-expressing vessels in normal and neoplastic tissue, there also seems to be a stronger staining in tumors. This may be due to a higher αvβ3 expression or a greater exposure of the LM609 epitope. Although staining intensities can be influenced by interassay variation, single sections showing normal and neoplastic areas in the same section confirmed the differences in amount and intensity of staining.

In breast carcinomas, αvβ3 expression revealed by the use of LM609 has been described on all the tumor vasculature of the lesions (Brooks et al., 1995). Here, the vascular αvβ3 expression in breast carcinomas ranged from 40% to 70%. This might be due to the marker defining blood vessels. A major reference antibody used in the literature is directed to the factor-VIII-related antigen. Unlike the PAL-E antibody, these antibodies often fail to stain tumor capillaries (Schlingemann et al., 1991) and will, therefore, underestimate the actual (micro-) vessel density.

In some carcinomas the vasculature within and surrounding inflammatory infiltrates showed more and more intensely stained vessels compared to the rest of the section. As tumor-induced angiogenesis may be initiated by release of angiogenic factors from tumor and inflammatory cells (Leek et al., 1994), this appears to indicate that angiogenesis might be enhanced by inflammatory cells.

Hypoxia may also lead to neovascularization. We saw single carcinomas with necrotic, presumably hypoxic, regions surrounded by many αvβ3-expressing vessels. This may indicate hypoxia-induced upregulation of vascular αvβ3 expression comparable to what has been reported for VEGF expression in human glioblastomas. There, tumor cells in immediate proximity to necrotic foci but not in non-necrotic areas produce VEGF, a known angiogenic cytokine (Shweiki et al., 1992).

In summary, our data confirm and extend earlier reports on the upregulation of αvβ3 expression on the tumor vasculature. However, they also show that αvβ3 expression is not limited to areas of neovascularization and that the expression in normal tissue does not appear to be due to hypoxia or to a tissue-sampling artefact.
Unlike previous work (Mechtersheimer et al., 1994; Luscinskas and Lawler, 1994) anti-αvβ3 antibodies rather than single-chain specific anti-αv or β3-antibodies were used for our analysis and endothelial αvβ3 expression was more rigorously differentiated from αvβ3 expression on the media.

There are as yet no clues regarding the function of αvβ3 expression in normal tissue. This might indicate angiogenic activity from αvβ3 expression on the media. Specific anti-αv or p3-antibodies were used for our analysis and anti-αvβ3 antibodies rather than single-chain antibodies.

REFERENCES


