

# **Vascular density and endothelial cell expression of integrin alpha v beta 3 and E-selectin in murine tumours**

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Keywords: mouse tumours; vascularization; integrin alpha v beta 3; E-selectin; melanoma; colon; mammary; pancreas; sarcoma; lung

Abbreviation used: CAM, endothelial cell adhesion molecule.

## Abstract

The endothelial cell adhesion molecules, including the integrin alpha v beta 3 ( $\alpha v\beta 3$ ) and E-selectin, are involved in the process of angiogenesis required for tumour growth, cell migration and metastasis. The purpose of this study was to assess and compare widely used tumour models to select the ones most suitable for angiogenesis research. Fifteen murine tumours were selected including melanoma (B16), colon (C26, C38, C51), mammary (MA13, MA16, MA16/Adr, MA17, MA17/Adr, MA25, MA44), pancreatic (PO2, PO3), Glasgow osteogenic sarcoma (GOS) and Lewis lung carcinoma (LLC). The tumour vascular density, assessed using the platelet endothelial cell adhesion molecule 1 (PECAM-1; CD31) immunostaining, revealed that B16 melanoma was poorly vascularized (<5%), whereas the colon and mammary tumours were well vascularized (5-15%). The most vascularized tumours (>15%) were the pancreatic tumours (PO2 and PO3), the sarcoma (GOS) and the lung tumour (LLC). The integrin  $\alpha v\beta 3$  and E-selectin evaluated by immunohistology, showed that 7/15 tumours expressed the  $\alpha v\beta 3$  integrin which was homogeneously distributed on all tumour sections (B16, C26, MA17/Adr, MA25, MA44, PO2, LLC). E-selectin was expressed in 4/15 tumours and its expression was restricted to the tumour periphery. Only 2/15 tumours (B16 and C26) were shown to express both integrin  $\alpha v\beta 3$  and E-selectin. In conclusion, these data not only contribute to a better understanding of the tumour biology of murine tumours, but can also guide the choice of appropriate models for antiangiogenic therapy, for selective drug delivery to tumours and the validation of tumour imaging modalities targeting these endothelial cell adhesion molecules.

## Introduction

Angiogenesis, which is required for the growth of solid tumours and the metastatic dissemination of tumour cells, is probably the most important parameter for the control of cancer progression [1]. Angiogenesis is the formation of new capillaries from pre-existing microvessels and consists in a multi-step process, in which endothelial cells degrade their extracellular matrix, migrate into the perivascular spaces, proliferate and align to form cell-cell contacts to construct patent blood vessels (reviewed in [2]). This process is mediated by endothelial cell adhesion molecules (CAMs) represented by four principal classes: the selectins, which are transmembrane proteins subdivided in three groups E, L and P, expressed by endothelial cells, leucocytes and platelets, respectively; the integrins, which are transmembrane glycoproteins composed of two subunits  $\alpha$  and  $\beta$ ; the membrane proteins immunoglobulin superfamily; and, the cadherins, mediators of cell-cell interactions [3]. These CAMs also play an important role in the process of metastasis development and migration [4, 5]. Compared to normal tissues, the expression pattern of the different CAMs vary widely between tumour types [6]. Therefore, the knowledge of specific CAM expression in tumours is of importance because it can allow a better understanding of the biology of tumour metastasis and also contribute to better predict the tumour evolution *in vivo*.

Among the CAMs, two proteins appear more particularly involved in the angiogenesis process, i.e., the integrin alpha v beta 3 ( $\alpha v\beta 3$ ) and the E-selectin {Varner, 1996 10294 /id;Kraling, 1996 9166 /id}. The integrin  $\alpha v\beta 3$  is not (or weakly) constitutively expressed in normal vascular endothelial cells, whereas it is selectively expressed on proliferating vascular endothelial cells [9] and on vascular cells in tumours [10-12]. Several studies have indeed reported the requirement of integrin  $\alpha v\beta 3$  for angiogenesis {Brooks, 1994 10300 /id;Eliceiri, 1999 10301 /id;Weis, 2011 10351 /id}. Similarly, E-selectin is also detected at very low

levels in normal adult blood vessels, but is highly expressed in newly formed tumour capillaries [15] and in activated endothelium [4, 8]. In addition, several investigators have shown that E-selectin is involved in angiogenesis [8, 16, 17]. By their angiogenic and adhesion properties, both CAMs are involved in tumour growth and metastasis and can therefore influence cancer prognosis and/or response to antiangiogenic therapy [4, 5, 18, 19].

Although the importance of angiogenesis has become central to a better understanding of the cancer process, as well as to the search for more specific treatments, it is surprising that most preclinical murine models are poorly characterized with regard to their vascular density and their expression of the principal CAMs. This information could be helpful in order to select the best tumour models for angiogenic studies and to follow experimental therapeutic approaches.

The aim of the present study was to evaluate the vascular density of 15 frequently employed murine solid tumours using PECAM-1 (a constitutively expressed CAM; CD31), and to characterize the expression pattern of the integrin  $\alpha\text{v}\beta\text{3}$  and E-selectin using immunological methods. Our results show that each tumour type possesses a unique expression pattern of CAMs that could influence the tumour biology and response to therapy.

## Materials and methods

### *Mouse tumours*

Mouse tumours were obtained from the Aventis Oncology Department (now Sanofi, Vitry sur Seine, France) and were maintained in the mouse strain of origin. Briefly, tumour fragments (30-60 mm<sup>3</sup>) were implanted subcutaneously using a 12 gauge trocar into the flanks of 6-week-old female mice of the appropriate mouse strain (reviewed in [20]) for a given tumour, as listed hereafter: B16 melanoma was implanted in C57Bl/6 mice [21]; colon carcinoma C26 and colon adenocarcinoma C51 in Balb/C, and C38 in C57Bl/6 [22, 23]; mammary adenocarcinomas MA13 in Balb/C, MA16/C [24], MA16/C/ADR [25], MA17, and MA17/Adr in C3H/HeN mice [26], and MA25 and MA44 in C57Bl/6; pancreatic ductal adenocarcinomas PO2 and PO3 in C57Bl/6 mice [27]; Glasgow osteogenic sarcoma GOS in C57Bl/6 mice [28]; and, Lewis lung carcinoma LLC in C57Bl/6 mice [29]. Mice were sacrificed by cervical dislocation when the tumour size reached 200 mm<sup>3</sup>, i.e., 10-20 days post-inoculation depending on tumour type. Samples of tumours (5×5×5 mm) were embedded frozen in isopentane immersed in liquid nitrogen and stored at -70°C until cutting on microtome and prepared for immunohistological analysis.

### *Primary antibodies used for immunostaining*

Monoclonal rat antibodies anti-PECAM-1 (CD31, clone: MEC13.3), anti-E-selectin (CD62E, clone: 10E9.6) and hamster antibody anti-integrin  $\alpha v \beta 3$  (CD61, clone: 2C9.G2, directed against sub-unit  $\beta 3$ ) were obtained from BDBiosciences (Le Pont de Claix, France). These antibodies were shown to cross-react with the corresponding mouse antigens. The working dilutions for immunostaining were 50-fold for anti-PECAM-1 (15.6  $\mu\text{g/mL}$ ) and 20-

fold for anti-E-selectin (62.5 µg/mL) and anti-integrin  $\alpha\beta3$  (250 µg/mL). Omission of the primary antibody was used for negative controls.

#### *Immunohistological analysis*

Ten-micron frozen tissue sections were placed on Superfrost Plus slides. Immunostaining of the CAMs was performed using a three-step procedure as previously described [8]. Endogenous peroxidase was inactivated with 0.3% H<sub>2</sub>O<sub>2</sub> in phosphate-buffered saline (PBS) for 30 min. Non-specific binding sites were blocked with 10% serum of the species used for the secondary antibody in PBS for 30 min. Slides were then incubated for 1 h (CD31, anti-PECAM-1) or 2 h (CD62E, anti-E-selectin and CD61, anti-integrin  $\alpha\beta3$ ) at 37°C in a humidified chamber with primary antibody. After 3 rinses of 5 min each, slides were incubated for 30 min with the corresponding biotinylated-secondary antibody (dilution 1/400 for the goat anti-rat antibody (0.5 mg/mL) and 1/200 (125 µg/mL) for the mouse anti-hamster antibody). After 3 rinses, slides were re-incubated with the streptavidin-conjugated peroxidase (Sigma-Aldrich, St. Quentin Fallavier, France) according to the manufacturer's instructions (dilution 1/400). The DAB (3,3'-diamino-benzidine, Sigma-Aldrich) substrate was then added for 5 to 7 min until a brown precipitate was visible. Tissues were counterstained with Gill's hematoxylin (Sigma-Aldrich) and treated with 30 mmol/L NH<sub>4</sub>OH to generate a blue nuclear stain. Slides were dehydrated in graded ethanol solutions and xylene and mounted with Eukitt® (Sigma-Aldrich).

#### *Immunostaining semi-quantitative analysis*

The density of CAMs was expressed as a percent range of total cells, as follows: -, no staining detected; <1%, few positive cells; 1-5%, some positive cells in all tumour sections; 5-

15%, positive cells observed in all fields; >15%, high density of positive cells in all fields.

The immunostaining was assessed by two independent investigators in a blinded manner.

## Results

### *Choice of tumour types*

In this study, we investigated 15 solid murine tumours to evaluate their vascular density and to assess their expression of important molecular markers involved in angiogenesis. The tumours were chosen among the most frequently used mouse tumour models, and also to be representative of the most frequent human tumour types. The murine tumours examined are listed in Table 1 and included 1 melanoma, 3 colon carcinomas, 7 mammary adenocarcinomas, 2 pancreatic tumours, 1 osteosarcoma and 1 lung carcinoma. Along with the tumour code names are presented the mouse strain used for propagation and the tumour main characteristics including doubling time, histological type and metastasis potential (invasiveness).

### *Tumour vascular density*

Medium sized tumours of approximately 200 mm<sup>3</sup> were harvested and analyzed for the endothelial expression of PECAM-1 (CD31), which correlates with vascularization. Figure 1 illustrates the three different types of vascular density observed. The B16 melanoma presented a poor vascular density (<5%), whereas colon C26 (5-15%), and pancreatic adenocarcinoma PO2 (>15%) were well and highly vascularized, respectively. Table 2 presents the comparison of the vascular density for the 15 tumours investigated. It is noteworthy that only the B16 melanoma was poorly vascularized, whereas all the colon and the mammary tumours were well vascularized (5-15%). The pancreatic adenocarcinomas (PO2, PO3), sarcoma (GOS) and the lung carcinoma (LLC) were the most vascularized tumours in our study (>15%).

### *Tumour vascular distribution*

The vascular distribution pattern of each tumour type is presented in Table 2. Most tumours showed a homogeneous and regular distribution of blood vessels (colon, pancreas, sarcoma and lung). However, two tumour types presented a different profile: the B16 melanoma presented small spots with numerous vessels; and, all the mammary tumours showed a heterogeneous distribution of blood vessels.

### *Expression of integrin $\alpha\beta3$ in tumours*

The percent expression of positive vessels for integrin  $\alpha\beta3$  is presented in Table 2. Seven tumours expressed the  $\alpha\beta3$  integrin, although at various levels. The B16 melanoma, one colon tumour (C26), 3 mammary tumours (MA17/Adr, MA25, MA44), one pancreatic tumour (PO2) and the Lewis lung carcinoma expressed this integrin. However the  $\alpha\beta3$  integrin was expressed at higher levels in the Lewis lung carcinoma and the PO2 pancreatic adenocarcinoma. Representative photographs depicting a low expression (<5%) of  $\alpha\beta3$  in B16 melanoma, MA17/Adr, MA25 and MA44 mammary adenocarcinoma are shown in Fig. 2, whereas high expression (5-15%) is shown for the PO2 pancreatic adenocarcinoma and LLC Lewis lung carcinoma.

The distribution pattern of this integrin was however different for the expressing tumours. The MA17/Adr, MA25 and MA44 mammary adenocarcinomas expressed the  $\alpha\beta3$  integrin on some vessels distributed all over the tumour section, whereas the C26 colon carcinoma and B16 melanoma showed  $\alpha\beta3$  restricted to some areas localized at the tumour periphery. The high expression of this integrin in the LLC lung carcinoma and the PO2

pancreatic adenocarcinoma was however distributed differently: in the LLC,  $\alpha\text{v}\beta\text{3}$  was homogeneously distributed, whereas in PO2, the expressing cells formed clusters.

#### *Expression of E-selectin in tumours*

The E-selectin expression in the various tumours is presented in Table 2. E-selectin was detected in only four tumours (B16, C26, C51, PO3). Representative photographs are presented in Fig. 3, where MA17/Adr and PO2 did not show detectable levels of E-selectin. In B16 melanoma, only few positive cells showed E-selectin expression (Fig. 3), similarly to the C51 colon adenocarcinoma and the PO3 pancreatic adenocarcinoma which also showed some isolated vessels expressing E-selectin. The C26 colon carcinoma expressed E-selectin to a high degree (5-15%), with numerous vessels expressing this selectin, although with a heterogeneous distribution, as depicted in Fig. 3.

#### *Co-expression of integrin $\alpha\text{v}\beta\text{3}$ and E-selectin in tumours*

To study the localization of  $\alpha\text{v}\beta\text{3}$  and E-selectin in the two tumours that co-expressed these CAMs, i.e., B16 and C26, we used adjacent histological sections presented in Fig. 4. The co-expression of  $\alpha\text{v}\beta\text{3}$  and E-selectin was not homogeneously distributed and was only observed at the tumour periphery for B16 and C26 tumours. In addition, we noted that the detection of PECAM-1 expression exhibited a denser vascularization in these areas.

## Discussion

The aim of this study was to better characterize the vascular density and the CAMs expression in murine solid tumours frequently employed in preclinical research. These data could facilitate the choice of the best tumour models for testing therapeutic approaches aiming at blocking angiogenesis or destroying existing tumour vascularization.

Concerning the vascular density, assessed with the staining of a CAM constitutively expressed by endothelial cells (PECAM-1, CD31), we found significant differences between the various tumours examined. Indeed, the least vascularized tumour was the B16 melanoma, whereas the colon and mammary tumours were all well vascularized. Also of note, the pancreatic tumours, the sarcoma (GOS) and the lung (LLC) tumours were found to be the most vascularized tumours in our series. There was apparently no correlation between the vascular density and the metastatic potential, because some poorly vascularized tumours are known to be highly metastatic (e.g., B16; compare Tables 1 and 2), whereas other tumours were highly vascularized and are moderately metastatic (e.g., GOS). Although these results may appear to contradict observations showing that high vascularization often correlates with metastatic potential within the same tumour type (reviewed in [30]), it should be noted that in our study, different tumours from various tissue origins were compared, and not at various growth stages for the same tumour type. Also, the results showing poor vascularization for the B16 melanoma could support the hypothesis that a tumour hypoxic environment could favour metastasis as previously reported [31].

The vascular distribution pattern in tumours was also of interest, because it revealed a well-organized vascularization in all subcutaneously transplanted tumour models. Most tumour types presented a regular and homogeneous distribution of blood vessels (colon, pancreas, sarcoma and lung). However, in the B16 melanoma and the mammary tumours, the

blood vessel distribution was found heterogeneous. For the mammary tumours, it was noteworthy that tumours of a same organ origin exhibited a similar vascularization pattern, even if they were transplanted in different mouse strains.

In humans, high tumour vessel density has been shown to be associated with metastasis, tumour progression and decreased survival time in several tumour types, including breast, lung, melanoma, colon, cervix, prostate and bladder [32, 33]. Although increased vascularity could be anticipated to favor oxygenation and drug delivery to tumours, it is possible that the more vascularized tumours metastasize to distant sites and therefore become more difficult to treat because of their dissemination throughout the organism [33].

In this study, we have also assessed the expression of two endothelial CAMs involved in angiogenesis in frequently employed mouse tumour models. These CAMs, including the integrin  $\alpha\text{v}\beta\text{3}$  and the E-selectin, are particularly important molecules because they are involved in the process of angiogenesis which is required for tumour growth, cell migration and metastasis {Weis, 2011 10351 /id}.

Integrin  $\alpha\text{v}\beta\text{3}$  is not constitutively expressed by normal endothelial cells and is therefore considered diagnostic of proliferative endothelial cells in neovascularized areas [9]. In the present study, the  $\alpha\text{v}\beta\text{3}$  integrin was expressed in 7/15 of the tumours examined (B16, C26, MA17/Adr, MA25, MA44, PO2, LLC). It is noteworthy that there was no correlation between the tumour type and the expression of this integrin because only 1/3 of colon tumours and 3/7 of mammary tumours expressed this integrin. For the distribution pattern of this integrin in expressing tumours, it was interesting to observe that mammary and lung tumours have a homogenous distribution, whereas C26 and B16 melanoma presented a peripheral distribution. Similar results obtained by magnetic resonance imaging have recently been published for the B16 melanoma where it was also observed that  $\alpha\text{v}\beta\text{3}$  is peripherally distributed [34]. Similarly to mouse tumours, the integrin  $\alpha\text{v}\beta\text{3}$  has also been found expressed

in the endothelium of several human tumours including melanoma, breast, colon, prostate, cervix, brain, and pancreas, colon, and lung [11, 19, 35, 36].

E-selectin was expressed in only 4 mouse tumours, including B16 melanoma, C26 and C51 colon tumours and pancreatic PO3 tumours. In the C26 colon tumour and B16 melanoma, E-selectin expression was restricted to areas localized at the tumour periphery, which suggests that these areas correspond to tumour neovascularization zones which also expressed the  $\alpha v\beta 3$  integrin. This localized expression of E-selectin at tumour periphery has already been observed in previous studies [37, 38]. Like integrin  $\alpha v\beta 3$ , E-selectin is not expressed by normal endothelial cells but was found on microvessels that showed proliferating endothelial cells [8]. With regard to the E-selectin clinical applications, antagonists have been developed to target cellular interactions with this CAM including antibodies, ligand inhibitors and metabolic carbohydrate mimetics [39]. E-selectin has also recently been used as a target for drug delivery [40].

We noticed that only half of the tumours expressed integrin  $\alpha v\beta 3$  and a quarter expressed the E-selectin. Previous studies have also reported heterogeneity in CAMs expression by tumour endothelium, i.e., some tumours are deficient while others highly express CAMs [6]. The deficient CAMs expression could be due to the fact that CAMs are also involved in the inflammatory responses and recruitment processes. A reduced endothelial CAM expression in tumour microvessels could facilitate tumour progression in avoiding the patrolling by circulating lymphocytes [6]. In addition, the heterogeneity of CAMs expression has been shown to depend on tumour type, implantation mode, growth site and host strain [41, 42]. In the present study, subcutaneously implanted tumours have been used and these models may not be the exact reflection of *in situ* tumours, for which more complex interactions between the different CAMs appear to regulate tumour angiogenesis [43].

As previously mentioned, tumour viability, growth and metastasis depend on tumour angiogenesis. Integrin  $\alpha\beta3$  and E-selectin mediate the processes of microvessel neoformation, and detection of the expression of both CAMs allows to determine whether angiogenesis occurs in a tumour. Indeed, several studies have reported the use of specific angiogenesis specific markers as targeting ligands for systemic drug or gene delivery to cancer [44-46] or to other vascular diseases [47].

The expression of these CAMs in tumours appears to be shared by murine and human tumours as well. For example,  $\alpha\beta3$  has indeed been found expressed in several human tumours, e.g., melanoma, breast, prostate, cervix, brain and pancreas [19, 35, 36]. E-selectin has also been identified in human melanoma as a novel target for inhibition of melanoma angiogenesis and tumour growth [53].

These CAMs can be the target of antiangiogenic therapy by using inhibitors of integrin  $\alpha\beta3$  [48, 49] or of E-selectin [16]. Indeed, a better knowledge of the CAMs expressed in tumours has already allowed the development of several therapeutic approaches. For example, integrin antagonists, including the  $\alpha\beta3$  and  $\alpha\beta5$  inhibitor cilengitide, have demonstrated encouraging activity in clinical trials [50, 51]. With regard to the E-selectin, antagonists have been developed to target cellular interactions with this CAM including antibodies, ligand inhibitors and metabolic carbohydrate mimetics [39]. E-selectin has also recently been used as a target for drug delivery [40].

In addition to therapeutic applications, the identification of these CAMs in tumours has also permitted the use of this knowledge for molecular imaging. The integrin  $\alpha\beta3$  has been targeted for imaging purposes with near-infrared fluorescent dye-RGD peptide conjugates, their multivalent analogs, and nanoparticle conjugates [50, 52]. E-selectin has also been used as a target for molecular imaging [40].

In conclusion, the assessment of the vascular density and the expression of the important integrin  $\alpha\text{v}\beta\text{3}$  and E-selectin in a series of widely used murine solid tumour models has allowed the identification of several tumours expressing these CAMs. We have also identified two tumours expressing both  $\alpha\text{v}\beta\text{3}$  and E-selectin (B16 and C26). These data may prove useful for the choice of appropriate tumour models for the study of the biology of tumour angiogenesis, the evaluation of antiangiogenic therapies and the validation of tumour imaging modalities targeting these CAMs.

## **Acknowledgments**

We are grateful to the Oncology Department of Aventis Pharma, S.A. (now Sanofi, S.A.), for providing the murine tumour samples. This work was supported in part by Gencell S.A., the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Ecole Nationale Supérieure de Chimie Paris (ENSCP). We also thank the Institut National du Cancer for grant support to GGC (INCa, Boulogne Billancourt, France).

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**Table 1. Principal characteristics of the murine tumours employed in this study**

| <b>Tumour code name</b> | <b>Mouse strain</b> | <b>Td<sup>a</sup> (days)</b> | <b>Histological type</b>                                               | <b>Invasiveness<sup>b</sup></b>                                   | <b>References</b> |
|-------------------------|---------------------|------------------------------|------------------------------------------------------------------------|-------------------------------------------------------------------|-------------------|
| B16                     | C57BL/6             | 1.25-3.0                     | Epidermoid melanoma                                                    | Highly metastatic: >70% to lungs                                  | [20, 21, 54-56]   |
| C26 colon               | Balb/C              | 1.7-2                        | Colon carcinoma, undifferentiated                                      | Highly metastatic: >90% to lungs                                  | [20, 22, 23, 57]  |
| C38 colon               | C57Bl/6             | 2.5-4.0                      | Colon adenocarcinoma                                                   | Moderately metastatic: <30% to lungs                              | [22, 23, 57]      |
| C51 colon               | Balb/C              | 2.5-4.3                      | Colon adenocarcinoma, mucinous, drug insensitive                       | Moderately to highly metastatic: >80% to lungs and to lymph nodes | [22, 23, 26, 57]  |
| MA13/C                  | Balb/C              | 3.4                          | Mammary adenocarcinoma                                                 |                                                                   | [24, 55]          |
| MA16/C                  | C3H/HeN             | 1.3-2.0                      | Mammary adenocarcinoma                                                 | Highly metastatic: >80% to lungs and >30% to lymph nodes          | [20, 24, 57]      |
| MA16/C/Adr              | C3H/HeN             | 1.1                          | Mammary adenocarcinoma, multidrug resistant, PgP <sup>c</sup> negative |                                                                   | [25]              |
| MA17                    | C3H/HeN             | 1.0                          | Mammary adenocarcinoma                                                 |                                                                   | [26]              |
| MA17/Adr                | C3H/HeN             | 1.0                          | Mammary adenocarcinoma, multidrug resistant, PgP <sup>c</sup> positive |                                                                   | [26]              |
| MA25                    | Balb/C              |                              | Mammary adenocarcinoma                                                 |                                                                   | [58]              |
| MA44                    | C3H/HeN             | 2.0                          | Mammary adenocarcinoma                                                 | Highly metastatic: >90% to lungs                                  | [20, 55]          |
| PO2                     | C57BL/6             | 2.2                          | Pancreatic ductal adenocarcinoma, drug insensitive                     | Highly metastatic                                                 | [27]              |
| PO3                     | C57BL/6             | 2.2-3.5                      | Pancreatic ductal adenocarcinoma, drug insensitive                     | Metastatic                                                        | [27]              |
| GOS                     | C57BL/6             | 1.9                          | Undifferentiated osteogenic sarcoma (Glasgow)                          | Moderately metastatic: <15% to lungs                              | [28, 55, 57]      |
| LLC                     | C57BL/6             | 1-1.7                        | Lung carcinoma (Lewis)                                                 | Metastatic to lungs (mostly) and kidneys                          | [20, 29, 59]      |

<sup>a</sup> Td, tumour doubling time in days. <sup>b</sup> Metastases from subcutaneously implanted tumour fragment. <sup>c</sup> PgP, P-glycoprotein.

**Table 2: Murine tumour models vascular density and expression of endothelial cell adhesion molecules. <sup>a</sup>**

| <b>Tumour type and name</b> | <b>Vascular density <sup>b</sup></b> | <b>Vascular distribution</b>                                                | <b>Integrin <math>\alpha\beta 3</math> <sup>c</sup></b> | <b>E-selectin <sup>d</sup></b> |
|-----------------------------|--------------------------------------|-----------------------------------------------------------------------------|---------------------------------------------------------|--------------------------------|
| <i>Melanoma</i>             |                                      |                                                                             |                                                         |                                |
| B16                         | <5%                                  | Some small spots showing numerous vessels                                   | <5%                                                     | <5%                            |
| <i>Colon</i>                |                                      |                                                                             |                                                         |                                |
| C26                         | 5-15%                                | Homogeneous distribution of vessels, some small spots with numerous vessels | <5%                                                     | 5-15%                          |
| C38                         | 5-15%                                |                                                                             | -                                                       | -                              |
| C51                         | 5-15%                                |                                                                             | -                                                       | <5%                            |
| <i>Mammary</i>              |                                      |                                                                             |                                                         |                                |
| MA13/C                      | 5-15%                                | Heterogeneous vessels distribution                                          | -                                                       | -                              |
| MA16/C                      | 5-15%                                |                                                                             | -                                                       | -                              |
| MA16/C/Adr                  | 5-15%                                |                                                                             | -                                                       | -                              |
| MA17                        | 5-15%                                |                                                                             | -                                                       | -                              |
| MA17/Adr                    | 5-15%                                |                                                                             | <5%                                                     | -                              |
| MA25                        | 5-15%                                |                                                                             | <5%                                                     | -                              |
| MA44                        | 5-15%                                |                                                                             | <5%                                                     | -                              |
| <i>Pancreas</i>             |                                      |                                                                             |                                                         |                                |
| PO2                         | >15%                                 | Homogeneous vessels distribution, some small spots with numerous vessels    | 5-15%                                                   | -                              |
| PO3                         | >15%                                 |                                                                             | -                                                       | <5%                            |
| <i>Sarcoma</i>              |                                      |                                                                             |                                                         |                                |
| GOS                         | >15%                                 | Homogeneous vessels distribution                                            | -                                                       | -                              |
| <i>Lung</i>                 |                                      |                                                                             |                                                         |                                |
| LLC                         | >15%                                 | Homogeneous vessels distribution                                            | 5-15%                                                   | -                              |

<sup>a</sup> Three independent samples were evaluated for each tumour type by 2 independent investigators, giving similar results. Tumour volumes were approximately 200 mm<sup>3</sup>.

<sup>b</sup> The vascular density was assessed using PECAM-1 immunostaining (CD31 antibody).

<sup>c</sup> The expression of  $\alpha\beta 3$  was assessed using CD61 antibody.

<sup>d</sup> E-selectin was assessed using CD62E antibody.

## Legends for Figures

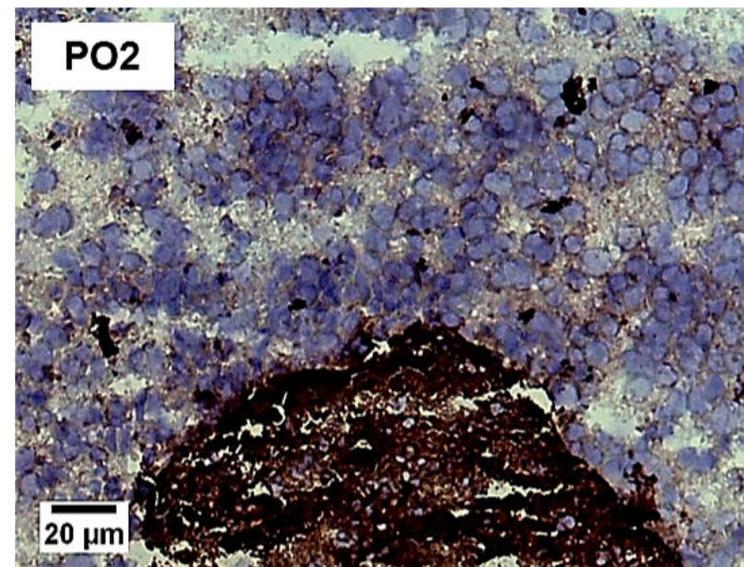
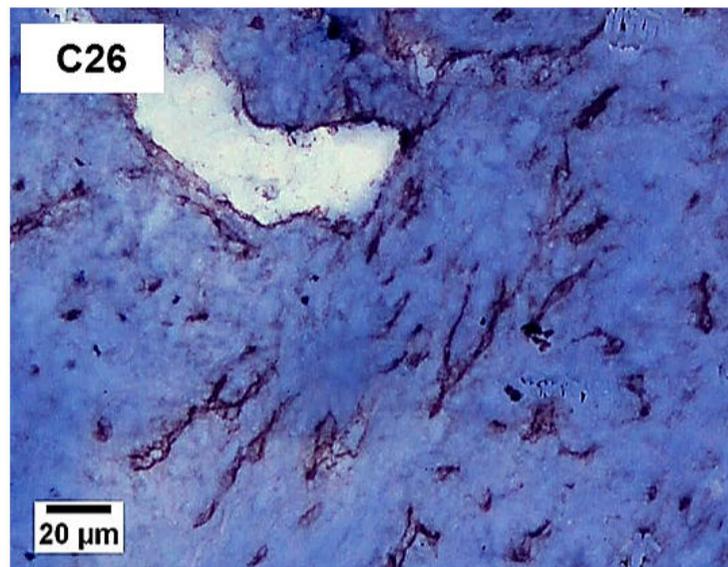
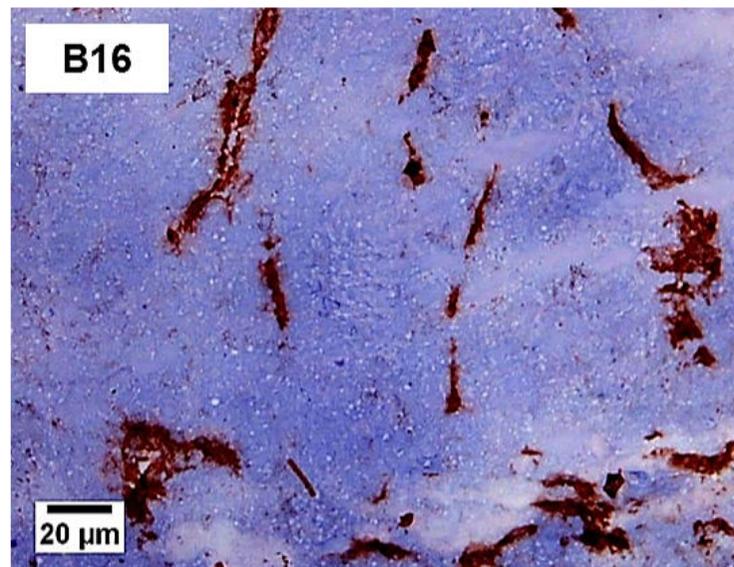
**Figure 1. Vascular density in representative mouse tumours.** B16 melanoma, poorly vascularized (<5%); C26 colon carcinoma, well vascularized (5-15%); PO2 pancreatic adenocarcinoma, highly vascularized (>15%). Mouse tumour slices of 10  $\mu\text{m}$  were prepared and stained using PECAM-1 (CD31) antibody as described in Materials and Methods.

Original magnification  $\times 100$ , bar 20  $\mu\text{m}$ .

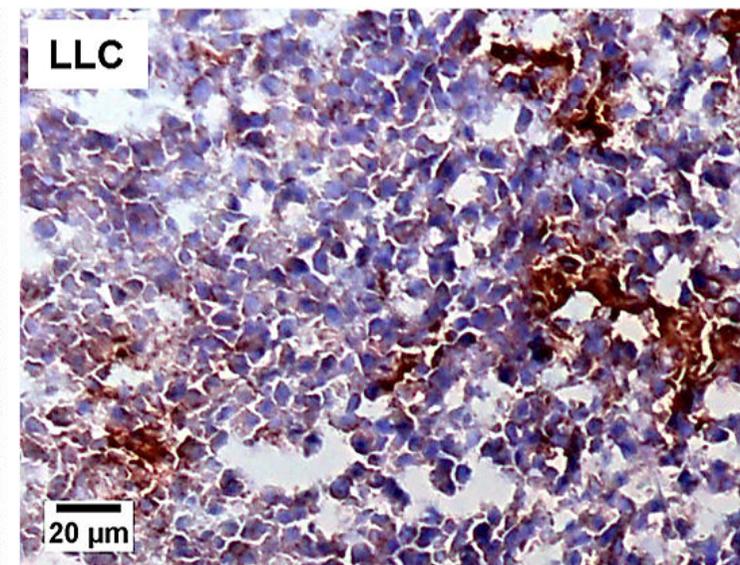
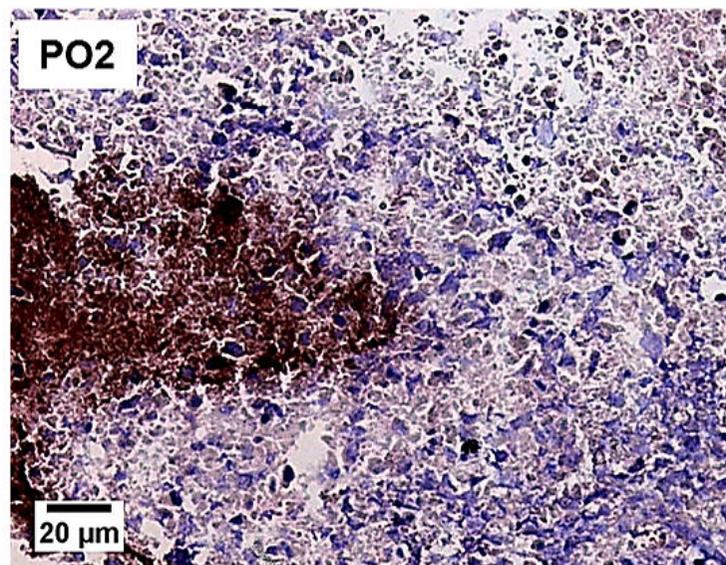
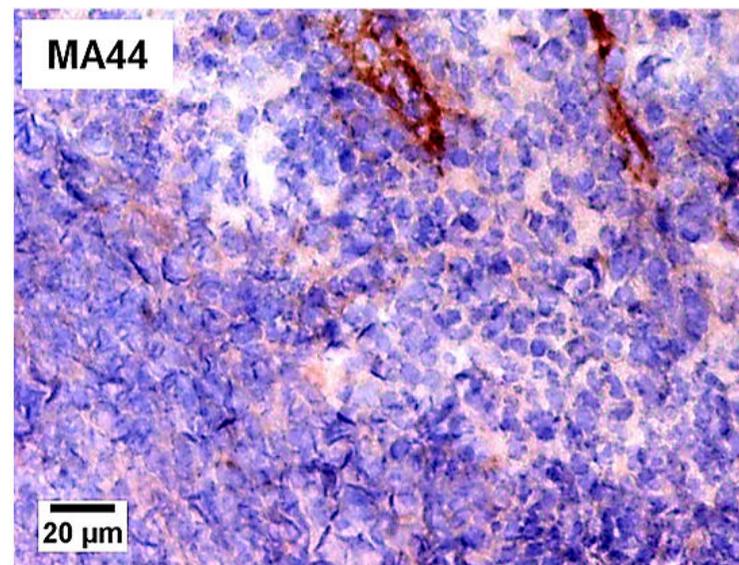
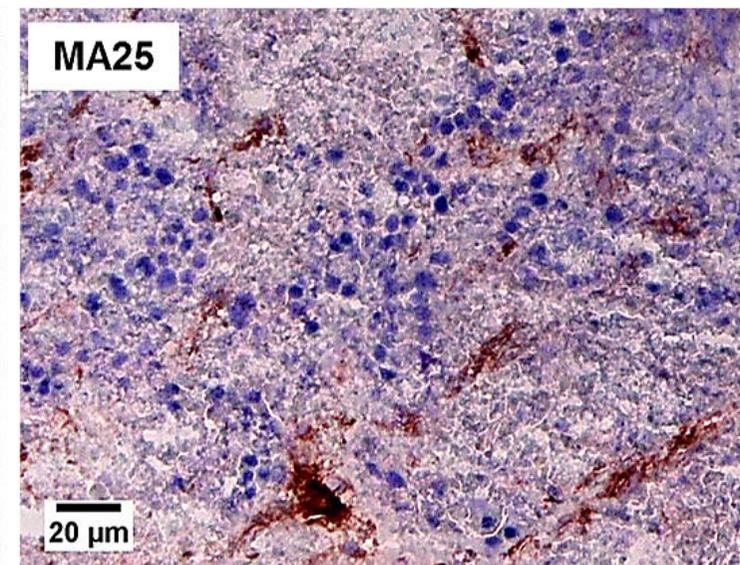
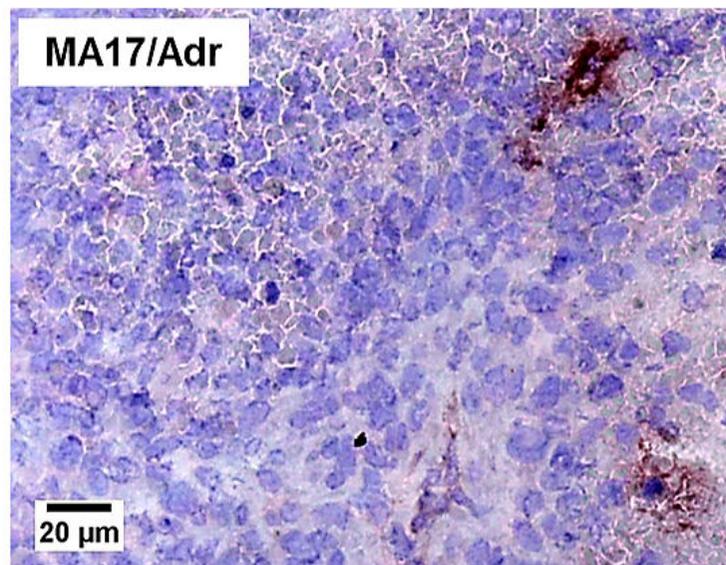
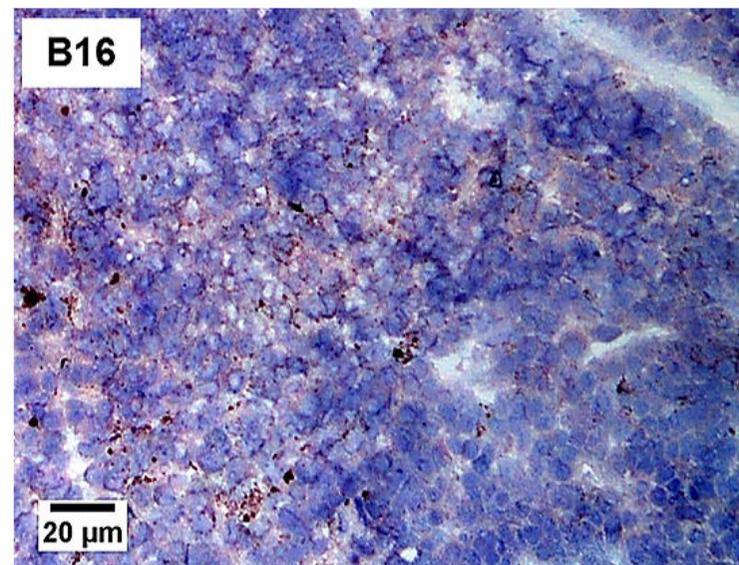
**Figure 2. Expression of the  $\alpha\text{v}\beta 3$  integrin in murine tumours.** B16 melanoma, MA17/Adr, MA25 and MA44 mammary adenocarcinoma show a low expression of the  $\alpha\text{v}\beta 3$  integrin (<5%), whereas PO2 pancreatic adenocarcinoma and LLC Lewis lung carcinoma presented a high expression (5-15%). Mouse tumours slices of 10  $\mu\text{m}$  were stained using CD61 antibody, as described in Materials and Methods. Original magnification  $\times 100$ , bar 20  $\mu\text{m}$ .

**Figure 3. E-selectin expression in representative mouse tumours.** MA17/Adr mammary adenocarcinoma and PO2 pancreatic adenocarcinoma representing undetectable expression; B16 melanoma, poor expression (<5%); C26 colon carcinoma, high expression (5-15%). Mouse tumour slices of 10  $\mu\text{m}$  were stained using CD62E antibody, as described in Materials and Methods. Original magnification  $\times 100$ , bar 20  $\mu\text{m}$ .

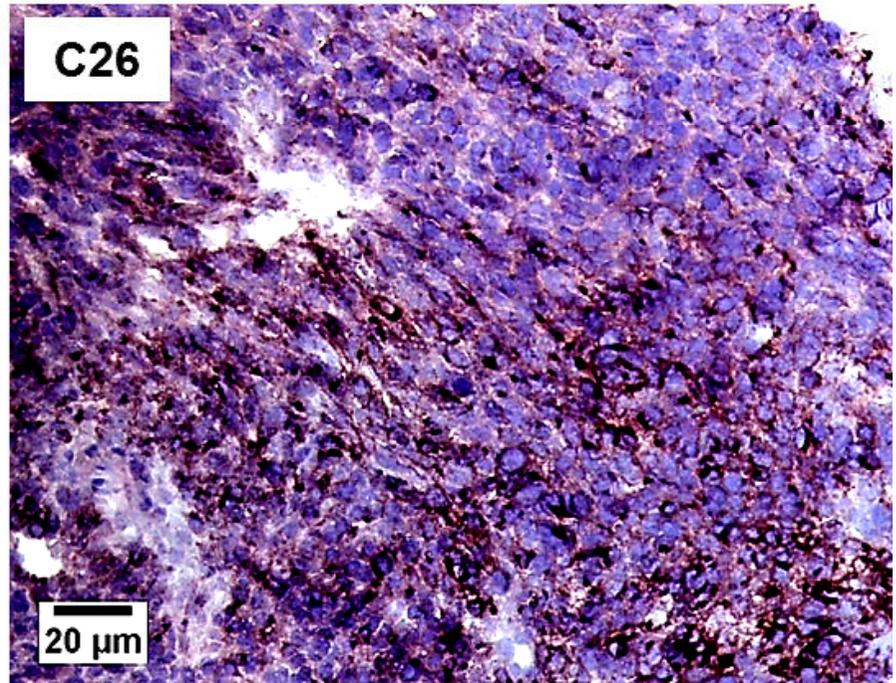
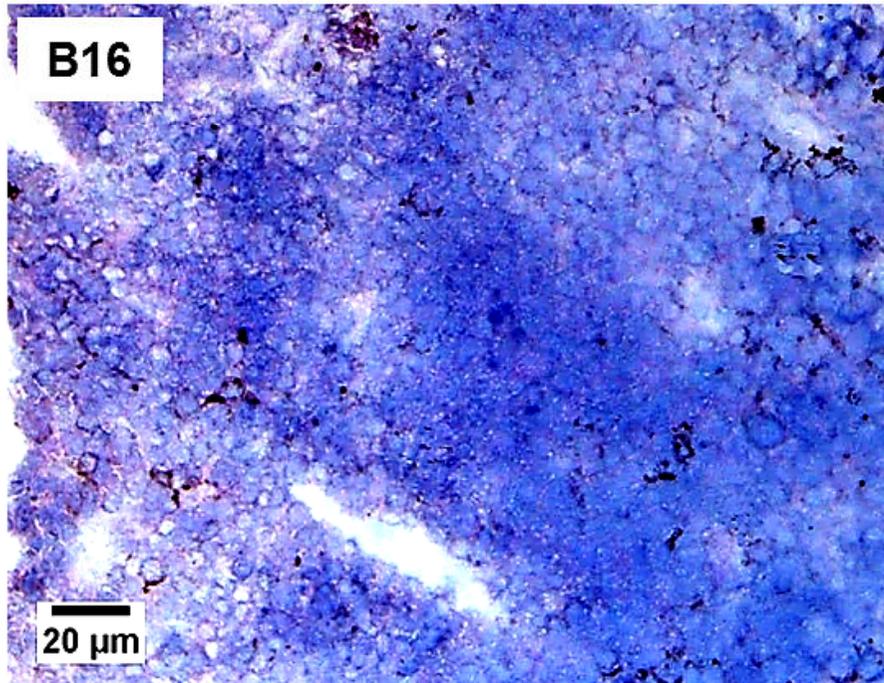
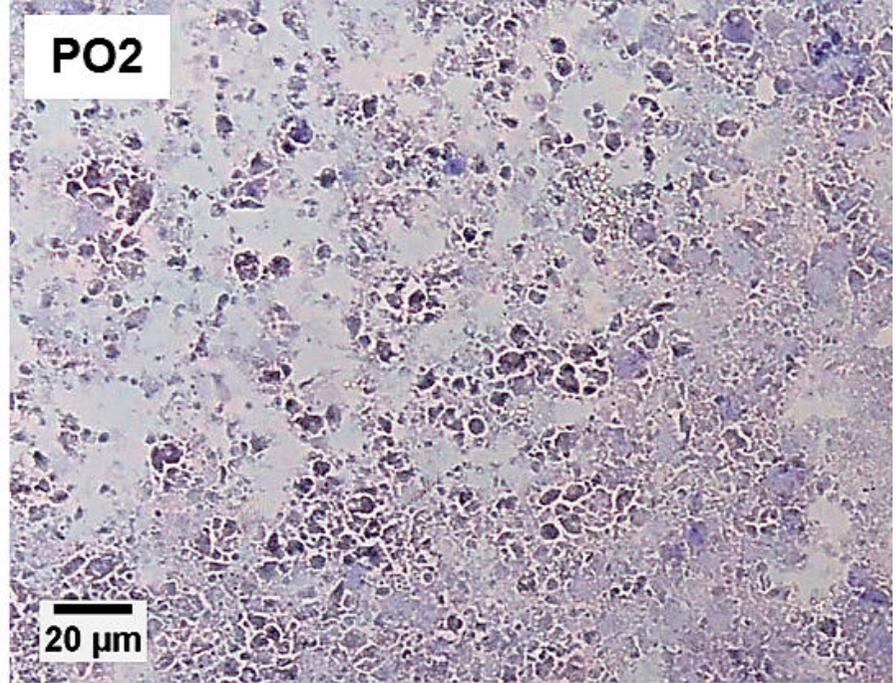
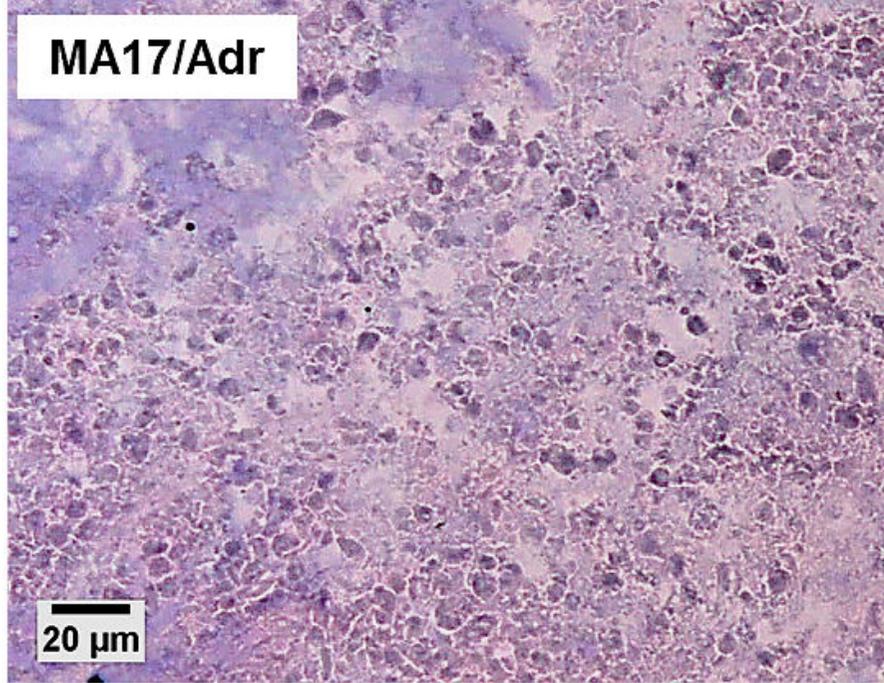
**Figure 4. Co-expression of  $\alpha\text{v}\beta 3$  integrin and E-selectin in colon carcinoma C26 and B16 melanoma.** Adjacent slices showing the vascularization (PECAM-1, CD31), the integrin  $\alpha\text{v}\beta 3$  (CD61) expression and the E-selectin (CD62E) expression in B16 melanoma and C26 colon tumour. Adjacent slices were processed with the different antibodies as described in Materials and Methods. Original magnification  $\times 100$ , bar 20  $\mu\text{m}$ .



**Fig. 1**



**Fig. 2**



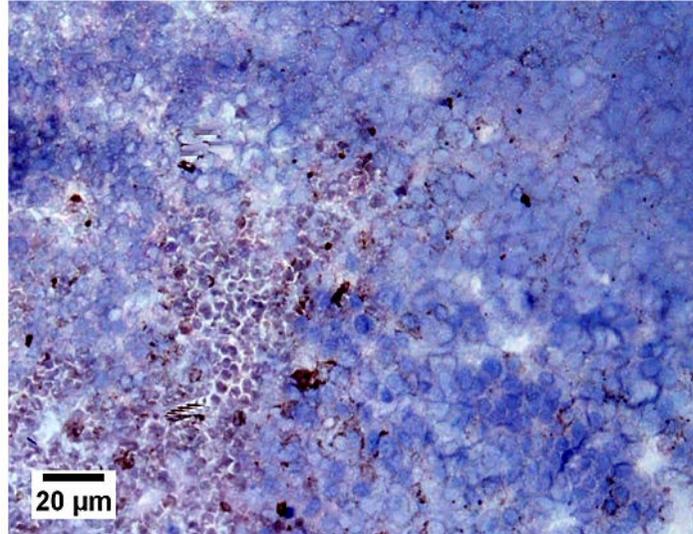
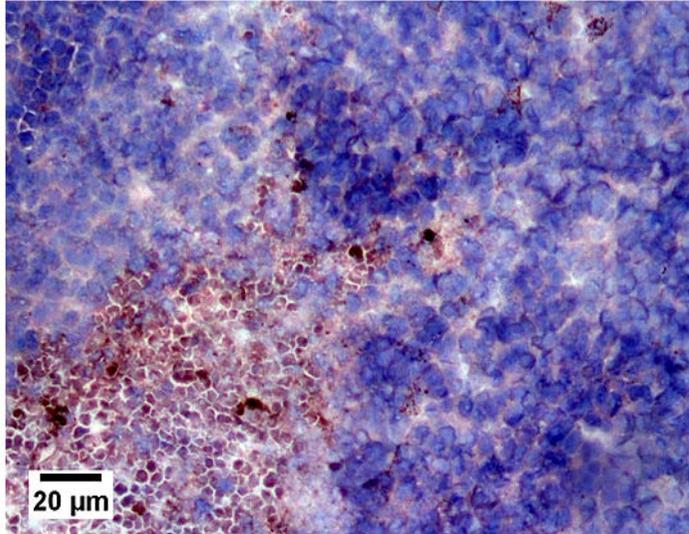
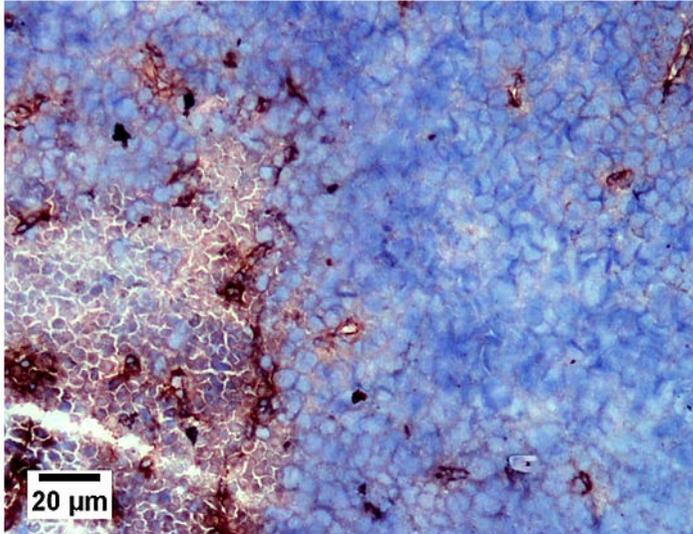
**Fig. 3**

**Vascularization**

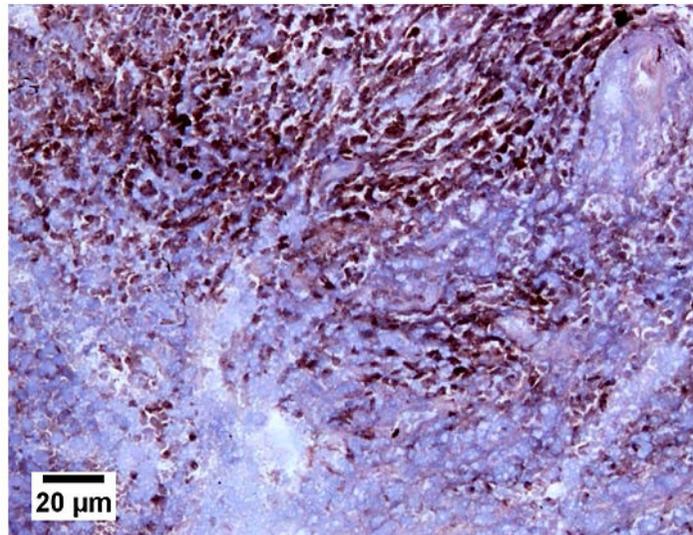
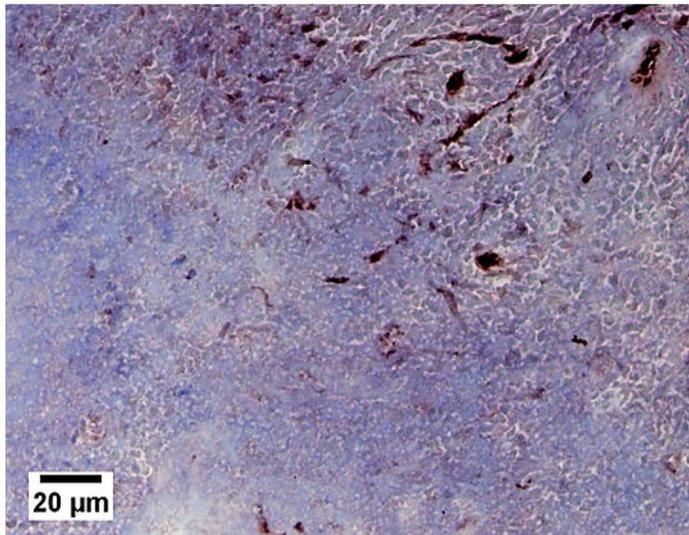
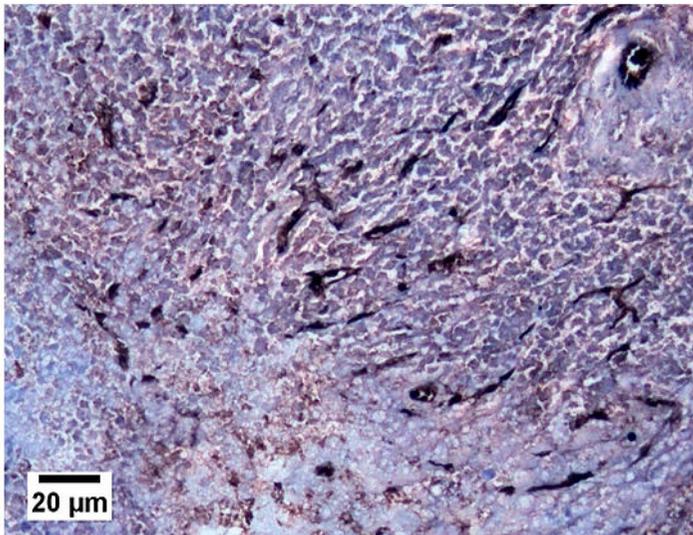
**Integrin  $\alpha v \beta 3$**

**E-Selectin**

**B16**



**C26**



**Fig. 4**