Virchows Arch (1999) 435:8–12

ORIGINAL ARTICLE

Jens-Ekkehard König · Edina Tolnay
Thorsten Wiethege · Klaus-Michael Müller

Expression of vascular endothelial growth factor in diffuse malignant pleural mesothelioma

Received: 5 January 1999 / Accepted: 9 March 1999

Abstract Angiogenesis is an important part of normal and pathological processes, including tumour growth, metastasis, inflammation and wound healing. VEGF is the best known angiogenic factor, implicated in tumour-associated microvascular hyperpermeability and carcinogenesis. We investigated 103 malignant pleural mesotheliomas, analysing the expression of vascular endothelial growth factor using immunohistochemistry and insitu hybridization. The grade of microvessel density was assessed with the aid of anti-factor-VIII antibodies. An increased expression of VEGF was found in biphasic and epithelioid mesotheliomas, correlating in a statistically significant manner ($P<0.042$). Insitu hybridization confirmed the specificity of VEGF mRNA expression. There was a robust correlation between VEGF expression and increased microvessel density ($P<0.001$), and positive mesotheliomas had significantly higher microvessel densities than negative specimens. There was also a significant correlation between microvessel density and histological pattern. As growth pattern tended towards biphasic and sarcomatoid mesotheliomas the density of micovessels decreased ($P<0.05$).

Key words Diffuse malignant pleural mesothelioma · Vascular endothelial growth factor · Factor VIII · Microvessel count · Histological differentiation

Jens-Ekkehard König worked on this study in partial fulfilment of the requirements for his M.D. degree, and the work is reported in his M.D. thesis

J.-E. König · T. Wiethege · K.-M. Müller (✉)
Institute of Pathology, University Hospital Bergmannsheil, Bürkle-de-la-Camp-Platz 1, D-44789 Bochum, Germany
e-mail: patho-bhl@ruhr-uni-bochum.de
Tel.: +49 234 302 6600, Fax: +49 234 302 6671

E. Tolnay
Department of Pulmonology, Semmelweis Medical School, Budapest, Hungary

Introduction

Angiogenesis is a an essential part of a variety of physiological and pathological processes, including carcinogenesis, metastasis, inflammation and wound healing. Angiogenesis implies neovascularization or formation of new blood vessels from pre-existing microvessels. Carcinogenesis is known to be dependent on neovascularization, which may be caused by an imbalance of angiogenic and antiangiogenic factors [10].

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is an peptide and is reported to be secreted by a variety of malignant tumours [2, 3, 5, 11, 14–18]. It is a potent inducer of microvascular permeability; on a molar basis it increases microvascular permeability with a potency some 50,000 times that of histamine. VEGF is a heparin-binding, dimeric glycoprotein with a selective mitogenic effect on vascular endothelial cells in vitro and a direct angiogenic effect in vivo [6, 9]. Different splicing of a single gene transcript results in four protein isoforms (121, 165, 189, and 206 amino acids) of the growth factor, with various biological activities. The largest isoform remains cell associated, owing to its greater affinity to heparin-containing proteoglycans located in the cell membrane [8]. Three high-affinity tyrosine-kinase receptors are identified for VEGF in humans: KDR (kinase domain receptor), *flt-1* (fms-like tyrosine kinase) and a soluble form of the VEGF *flt-1* receptor [16].

VEGF is synthesized and secreted by a variety of human tumours [8, 14]. It is accepted that malignant tumours have to induce a vascular stroma to grow beyond a minimal size.

Diffuse malignant mesotheliomas of any histological category grow by direct extension with encasement and later infiltration of the lung [12]. They are rare tumours, occurring in a large cases in patients with a history of exposure to asbestos [12]. Nevertheless, malignant mesotheliomas, like other tumours, must follow simple angiogenic rules. Recently we described an increased expression of hepatocyte growth factor/scatter factor and over-

expression of c-Met receptor in malignant mesotheliomas, closely correlated with increased microvessel density [18].

Less is known about function of VEGF in malignant mesotheliomas, and no information is available on the role of VEGF in malignant pleural mesotheliomas or its relationships to histological patterns.

The purpose of the present study was analyse 103 malignant pleural mesotheliomas with reference to expression of VEGF and the grade of microvessel density.

Materials and methods

One hundred and three formalin-fixed and paraffin-embedded biopsy (33), resection (11) and obduction (59) specimens from the archives of the German Mesothelioma Registry held in the Institute of Pathology were investigated. Sixty-nine specimens were classified as mesothelioma A and 34, as mesothelioma B according to the classification of the European Mesothelioma Panel [11]. There were 89 male and 14 female patients. Their median age amounted to 65 years (range 42–91).

Forty-six specimens were classed as epithelioid, 46 as biphasic, and 11 as of the sarcomatoid type [19].

Immunostaining of VEGF and factor-VIII expression was performed using the alkaline phosphatase-anti-alkaline phosphatase method (APAAP) [4] with a rabbit polyclonal antibody (Santa Cruz, Calif.). Sections of 4 μm were cut from formalin-fixed, paraffin-embedded tissue specimens and mounted on poly-L-lysine-coated slides. Paraffin sections were dewaxed by xylene, rehydrated and finally washed in Tris-Buffer (pH 7.6) for 10 min. VEGF required proteinase-K predigestion in a working solution of 0.4 mg/ml (Dako) for 10 min at room temperature. The following steps were optimized by an automatic staining system (Dako, TechMate 500). Sections were incubated with the primary antibody solution for VEGF at a dilution of 1:400 and factor VIII at a dilution of 1:30,000 for 25 min at room temperature. Slides were rinsed in buffer (Buffer Kit, Dako) and immunoreaction was completed with the APAAP kit (Dako). The secondary antibody was an alkaline phosphatase labelled monoclonal calf antibody, and the detection antibody a monoclonal anti-calf mouse antibody. After incubation with a chromogen alkaline phosphatase substrate (Fast Red, Dako) specimens were counterstained with Mayer's haematoxylin and coverslipped. The intensity of VEGF immunostaining was evaluated semiquantitively by light microscopy (Leitz). The amount of staining product was classified as showing no (-), slight (+), moderate (++) or strong (+++) immunoreactivity.

The grade of neovascularization was assessed by microvessel density, obtained by factor-VIII-related antigen staining that was specific for endothelial cells. Specimens' surface was quantified with an automatic image analysing system (LUCIA, Nikon). Total microvessels were assessed by light-microscopy. Microvessels were also counted in selected areas of highest neovascularization. Areas of highest neovascularization (hotspots) were found by scanning the tumour sections at low magnification (×40) and selecting the areas with the highest density of factor-VIII-staining

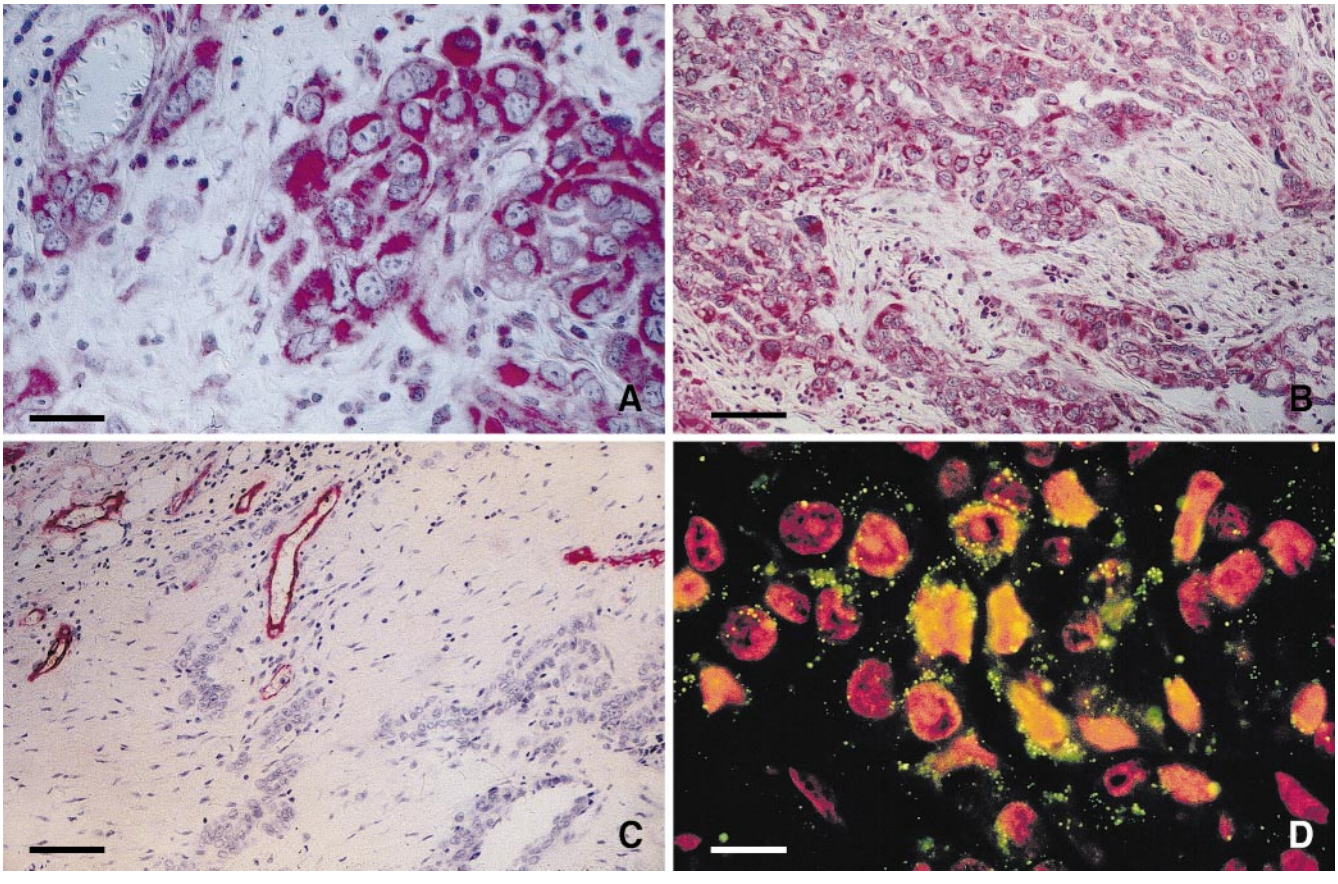

Fig. 1A–D Diffuse malignant pleural mesothelioma of epithelioid histological pattern. A–C Immunohistochemical staining for vascular endothelial growth factor (VEGF, A, B) and microvessels (factor-VIII, C). D Nonradioactive insitu hybridization of VEGF mRNA A Tumour cells and positively stained endothelial cells of the neighbouring microvessels. Anti-VEGF antibody, bar 20 μm. **B** Intense staining of malignant cells. *Bar* 50 μm C Factor-VIII microvessel staining close to tumour margin. *Bar* 50 μm D High intensity of VEGF mRNA expression confirmed VEGF immunostaining. Intense transcript signals in the cytoplasm. *Bar* 20 μm

microvessels. Individual hotspot counts were made on a ×100 field (×10 objective and ×10 ocular, Leitz). In each specimen three values were determined leading to a mean value of microvessel density. Measurement were all checked by a second pathologist. Microvessel count was assessed by a method described by Weidner in 1995. Analyses of the vessel luming proved not to be necessary for definition of a microvessels [20].

Insitu hybridization was carried out on formalin-fixed and paraffin-embedded tissue specimens as previously described [21].

Tissue samples were labelled by digoxigenin using the random primed oligolabelling method (Boehringer Mannheim, Germany). VEGF cDNA, a 225-bp fragment subcloned into the EcoRI/BamHI site of a pGEM 3 vector [2, 3], was a generous gift from Harold F. Dvorak (Harvard Medical School and Beth Israel Hospital, Department of Pathology, Boston, Mass.).

Tissue sections were analysed by a fluorescence microscope (Leica). Specific binding of the FITC conjugate to digoxigenin was monitored using hybridization solution without labelled probes during the hybridization step as control. The Spearman rank-order correlation test was applied for statistical analysis, with $P<0.05$ taken as level of significance.

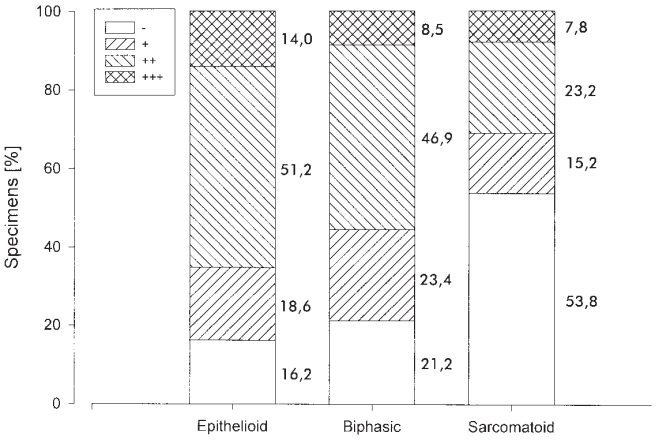

Fig. 2 VEGF immunostaining in different histological types of malignant mesotheliomas

Results

VEGF expression was localized in malignant tumour cells and in macrophages of alveolar spaces (Fig. 1A, B). Normal mesothelial cells showed no detectable VEGF expression. Endothelial cells of microvessels near the tumours stained positive with anti-VEGF antibody (Fig. 1A).

Expression of VEGF correlates with histological differentiation (Fig. 2). Most of the epithelioid specimens showed high or moderate immunoreactivity, whereas in sarcomatoid malignant mesotheliomas we detected only slight immunoreactivity or none at all ($P<0.042$). In total, 16 specimens stained strongly (+++) with anti-VEGF antibodies, and 16 specimens out of 103 showed moderate (++) immunoreactivity. Forty-four specimens (42.7%) were labelled positive (slight/+) for VEGF; 25 specimens (24.3%) showed no immunoreactivity for VEGF antibodies.

VEGF localization was confirmed by increased levels of VEGF mRNA staining (Fig. 1D). Malignant tumour cells and many of the activated macrophages in alveolar spaces labelled strongly for VEGF mRNA. VEGF immunostaining and VEGF mRNA expression could be specifically detected in the cytoplasm (Fig. 1D).

As VEGF expression increased the mean microvessel count also increased. The mean microvessel count in VEGF-negative specimens was 2.5 vessels/mm^2, but in specimens with strong VEGF immunostaining 27.0 vessels/mm^2 were found (Figs. 1C, 3). The correlation between these two variables was statistically significant (Spearman rank order test, $P<0.001$).

Simultaneously, areas of highest neovascularization (hotspots) were characterized by increased neovascularization in correlation with an increased degree of anti-VEGF antibody immunoreactivity ($P<0.0025$). These neovascular clusters might occur anythere within the tumour, but most frequently appeared at the margins (Fig. 1C). The hotspot vessel count in VEGF negative specimens was 24.0 vessels/mm^2, the corresponding value in the specimens with strong VEGF immunostaining being 82.1 vessels/mm^2 (Fig. 3.

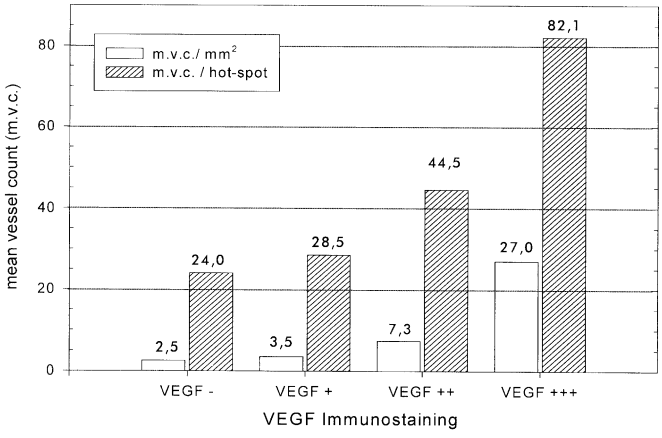

Fig. 3 VEGF immunostaining against mean microvessel count/mm^2 ($P<0.001$) and mean microvessel count/hotspot ($P<0.0025$)

There was a close correlation between growth pattern and mean microvessel count/mm^2 ($P<0.05$). Mesotheliomas with epithelioid growth pattern tended to higher microvessel densities than biphasic or sarcomatoid tumours. Mean vessel count of 10.4 vessels/mm^2 were found in epithelioid tumours, 7.5 vessels/mm^2 in biphasic and 5.9 vessels/mm^2 in sarcomatoid mesotheliomas.

Simultaneously, a close correlation between hotspots and growth pattern was found ($P<0.05$). Decreasing tumour differentiation was associated with decreasing hotspot microvessel densities.

Discussion

Angiogenesis is the formation of new blood vessels from the established microcirculation. Tumour growth and metastasis are angiogenic-dependent processes; a variety of normal and pathologic processes are characterized by

angiogenesis [8]. In recent years angiogenesis has become a focus of interest in carcinogenesis. Recently, it was shown that neovascularization takes place in the development of hyperplastic, metaplastic and preneoplastic lesions of the bronchial mucosa [7]. The ability to change during development from preneoplastic lesions to malignant tumours could be thought of as an imbalance of angiogenic and antiangiogenic proteins [10], and taking this into account neovascularization could be used to evaluate malignancy.

Anti-factor-VIII antibody is a good tool for differentiation between microvessels and other tissue components. Various studies have demonstrated a correlation between neovascularization assessed by factor VIII staining and metastasis [19], with a close correlation between manual and automatic intratumour microvessel determination [20].

In our study we observed a strong expression of VEGF mRNA and protein in different histological subtypes of malignant mesotheliomas, a close correlation of microvessel count and hotspots with expression of VEGF, and a significant correlation between histological differentiation and microvessel count.

Few data exist on the role of VEGF in malignant pleural mesotheliomas, but there are similar data for other tumours. Our data are consistent with the findings of Brown et al. [3] and Takahashi et al. [16], who demonstrated a strong correlation between VEGF expression and vessel count in tumours of the gastrointestinal tract.

We also obtained new data on the histological differentiation of malignant mesotheliomas, specifically on VEGF expression and microvessel count in epithelioid, biphasic and sarcomatoid tumour types. Expression of VEGF was significantly more pronounced in epithelioid tumours than in mesotheliomas with biphasic and sarcomatoid histological differentiation. Complementary microvessel count and the number of hotspots decreased simultaneously. Our results tie in with a recent study published by Kumar-Singh et al., who found increased expression of syndecan-1 in epitheloid mesotheliomas, whereas its expression was reduced or lost in biphasic or sarcomatoid differentiated tumours [13].

Less is known about the effect of histological pattern on tumour biology, but it seems that VEGF is more important in mesotheliomas with an epithelioid histological pattern than in biphasic or sarcomatoid types. As differentiation decreases and the tumour reaches an increasingly advanced stage, the growth pattern tends to change to that of biphasic and sarcomatoid mesotheliomas, with a greater frequency of necrosis and a decrease in the extent of inflammatory infiltrate [1].

Malignant mesotheliomas are rare tumours often associated with asbestos exposure. After a long period of latency, asbestos exposure and chronic irritation, diffuse malignant mesotheliomas of any histological category grow rapidly by direct extension with encasement. In advanced stages infiltration of the lung occurs [12]. Following years of irritation and inflammation, hyperplasia, metaplasia and preneoplasia occur in the pleural tissue. Increased neovascularization supports tissue remodelling, with the potential result of an angiogenic switch and the induction of neoplasia [7, 10].

It might be suggested that in most cases the long period of latency for tumour occurrence is responsible for the high vessel count at the point when the angiogenic switch occurs and the differentiation of tumour tissue begins to decrease. In this regard, the highest vessel count was found in specimens with epithelioid differentiation; Brockmann [1] showed that most malignant mesotheliomas were of the epitheloid type in the early stages.

More or less intense VEGF expression may reflect an attempt to provide sufficient microvessel growth to support malignant tumour cell proliferation. The greater frequency of necrosis in biphasic and sarcomatoid histological patterns may result in less prononced expression of VEGF and a consequent decrease in microvessel growth [11].

It is not clear at what point in oncogenesis the angiogenic switch occurs; it seens obvious that it happens during early tumour development. Recent data on enhanced neovascularization in hyperplastic, metaplastic and preneoplastic lesions of the bronchial mucosa confirm our observations [7].

The analyses of angiogenic activity may be an important step in examining tumour malignancy in future [13, 19]. It is unlikely that VEGF is the only angiogenic factor in malignant pleural mesotheliomas. Recently we have demonstrated that hepatocyte growth factor and its receptor cMet are overexpressed and associated with increased microvessel density in malignant mesotheliomas [18].

Our results emphasize a relationship between expression of VEGF, histological differentiation and vessel count. The studies have been extended to demonstrate a relationship between VEGF-associated receptors and vessel endothelium.

References

1. Brockmann M (1992) Malignes diffuses Pleuramesotheliom – Heterogenität, Differentialdiagnose und Histogenese. Hauptverband der gewerblichen Berufsgenossenschaften, Sankt Augustin
2. Brown LF, Berse B, Jackman RF, Tognazzi K, Manseau E, Dvorak H, Senger DR (1993) Increased expression of VPF and its receptors in kidney and bladder carcinoma. Am J Pathol 143:1255–1262
3. Brown LF, Berse B, Jackman RF, Tognazzi K, Manseau E, Dvorak H, Senger DR (1993) Expression of vascular permeability factor (VEGF) and its receptors in adenocarcinomas of the gastrointestinal tract. Cancer Res 53:4727–4735
4. Cordell JL, Fallini B, Erber WN (1984) Immunoenzymatic labeling of monoclonal antibodies using immunocomplexes of alkaline phosphatase and monoklonal anti-alkaline phosphatase (APAAP) complexes. J Histochem Cytochem 32:219–229
5. Dvorak HF, Sioussat TM, Brown LF, Berse B, Nagy JA, Janice A, Sotrel A, Manseau EJ, Van De Water L, Senger DR (1991) Distribution of vascular permeability factor in tumours: concentration in tumour blood vessels. J Exp Med 174:1275–1278
6. Ferrara N, Houck K, Jakema L (1992) Molecular and biological properties of the vascular endothelial growth factor family of proteins. J Endocrinol 13:18–32

7. Fisseler-Eckhoff A, Rothstein D, Müller K-M (1996) Neovascularization in hyperplastic, metaplastic and potentially preneoplastic lesions of the bronchial mucosa. Virchows Arch 429:95–100
8. Folkman J (1995) Tumour angiogenesis. In: Mendelson J (ed) The molecular basis of cancer. Saunders, Philadelphia, London, Toronto, pp 207–232
9. Goto F, Weindel K (1993) Synergistic effects of VEGF on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels. Lab Invest 69:508–514
10. Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumourigenesis. Cell 86:353–364
11. Jones JSP (1993) The formation and function of the mesothelioma panel of the Commission of the European Community. (Health and safety series, EUR 14522) Office for official publications of the European Communities, Luxembourg
12. Jones JSP, Brachet EA, Butler EB (1987) The pleura and its pathology. In: Jones JSP (ed) Pathology of the mesothelium. Springer, London Berlin Heidelberg, pp 39–133
13. Kumar-Singh S, Jacobs W, Dhaene K, Weyn B, Bogers J, Weyler J, Van Mark E (1998) Syndecan-1 expression in malignant mesothelioma – correlation with cell differentiation, WT1 expression and clinical outcome. J Pathol 186:300–305
14. Plate KH, Breier G, Weich HA, Risau W (1992) Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 359:845–848
15. Shweiki D, Itin A, Neufeld G (1993) Patterns of expression of VEGF and receptors in mice suggest a role in hormonally regulated angiogenesis. Clin Invest 91:2235–2236
16. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM (1995) Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. Cancer Res 55: 3964–3968
17. Tolnay E, Wiethege TH, Kuhnen C, Wulf M, Voss B, Müller K-M (1997) Expression of type IV collagenase correlates with the expression of VEGF in primary non-small cell lung cancer. Cancer Res Clin Oncol 123:652–658
18. Tolnay E, Kuhnen K, Wiethege T, König JE, Voss B, Müller KM (1998) Hepatocyte growth factor/scatter factor and ist receptor c-Met are overexpressed and associated with an increased microvessel density in malignant pleural mesothelioma. J Cancer Res Clin Oncol 124:291–296
19. Travis WD, Colby TV, Corrin B, Shimosato Y, Brambilla E (1999) Histological typing of lung and pleural tumors. World Health Organization. International histological classification of tumours. 3rd edn. Springer, Berlin, pp 51–54
20. Weidner N (1995) Intratumour microvessel density as a prognostic factor in cancer. Am J Pathol 147:9–19
21. Wiethege TH, Voss B, Pohle T, Fisseler-Eckhoff A, Müller K-M (1991) Localization of elastase and tumour necrosis factor alpha mRNA by non-radioactive in situ hybridization in cultures of alveolar macrophages. Pathol Res Pract 187: 912–915
22. Yeo K, Wang HH, Nagy JA, Sioussat TM, Ledbetter SR, Hoogewerf AJ, Zhou Y, Masse EM, Senger DR, Dvorak HF, Yeo T (1993) Vascular permeability factor (VEGF) in guinea pig and human tumour and inflammatory effusion. Cancer Res 53:2912–2918