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Expression of pro-angiogenic growth factors VEGF, EGF and bFGF and their topographical relation to neovascularisation in prostate cancer

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Abstract The aim of the study was to quantify the expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) in prostate cancer and adjacent non-tumorous tissue in a standardized experimental set-up and to evaluate the paracrine effects of three endothelial stimuli on neovascularisation. Immunohistochemical staining of prostate cancer (PCa) specimens for VEGF, bFGF, EGF and the endothelial marker CD31 was performed (n = 56). Sections were analyzed for growth factor-positive cancer/epithelial cells as well as staining intensity in (I) malignant and (II) non-tumorous tissue. Within PCa the topographic relationship (TR) of maximum microvessel density (MWD) and maximum expression of each growth factor was assessed. The number of VEGF- and EGF-positive cells in PCa was significantly enhanced compared with non-tumorous tissue ($p < 0.0001$), whereas there was no difference in staining intensity. In contrast, the staining intensity of bFGF sections revealed a stronger expression in non-tumorous tissue compared with PCa ($p < 0.0001$). In benign glands, VEGF, EGF and bFGF expression is chiefly restricted to basal cells. VEGF and EGF displayed a close TR in 65 and 57% of cases, respectively, whereas bFGF revealed a close TR in only 43% of PCa specimens. The results outline the relationship of the investigated growth factors and angiogenesis in PCa, which is strongest for VEGF and EGF. The relevance of VEGF and EGF is underlined by the increased number of positive cancer cells. Although previously reported to be a pro-angiogenic growth hormone, bFGF appears to play an assimilably minor role in the angiogenesis of PCa.

Keywords Prostate cancer · Angiogenesis · Growth factors

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Introduction

Angiogenesis plays a crucial role in the growth and metastasis of tumors, including prostate cancer [1, 2]. Angiogenesis is thought to be regulated by pro-angiogenic as well as anti-angiogenic influences [3]. Several peptides have been identified that promote angiogenesis and that are involved in the capillary differentiation of solid tumors. As previously described, some of the strongest are vascular endothelial growth factor (VEGF), the fibroblast growth factor (FGF) and the epidermal growth factor (EGF) [4, 5]. Several isoforms of VEGF and FGF exist [6, 7]. These growth factors are known to interact, e.g., in glioma cells VEGF expression can be stimulated by EGF and basic fibroblast growth factor (bFGF), a member of the FGF family [8]. Nevertheless, most knowledge on pro-angiogenic stimuli and angiogenesis of prostate cancer (PCa) has been acquired from cell culture and in vitro or animal models. So far, no trials with standardized experimental set-ups comparing different pro-angiogenic stimuli have been carried out.

Several therapeutic strategies targeting angiogenesis in prostate cancer have been developed [9]. Some of the most promising focus on the inhibition of angiogenesis-related receptors or receptor-agonist interactions [10, 11]. A prerequisite of such strategies, especially in clinical practice, is a thorough evaluation of the

pro-angiogenic growth hormones within the "target tissue" and the certainty that the proposed effector molecules are responsible for angiogenesis in the micromilieu of the individual carcinoma.

Our objective was to quantify the expression of three different pro-mitotic endothelial stimuli (VEGF, EGF and bFGF) in carcinoma and epithelial cells of human PCa specimens. The expression of peptides was evaluated (I) in a standard area (7.9 mm^2) of maximum expression within the carcinoma, (II) within the entire carcinoma of the investigated section, and (III) in surrounding non-tumorous tissue. Malignant and surrounding benign-appearing tissue served to elucidate the presence or absence of these growth factors.

Our second aim was to determine the paracrine effect of the three investigated pro-angiogenic stimuli on neovascularisation in PCa. A standard area of maximum microvessel density (MVD) in PCa was topographically related to the area of maximum expression of each of the stimuli. Our overall objective was to gain insight into the individual role of three known pro-angiogenic endothelial stimuli in human prostate cancer specimens.

Materials and methods

Patients and specimens

Our study included a total of 56 patients who underwent radical prostatectomy for clinically localized prostate cancer without prior therapy. The mean age of patients was 63.7 ± 6.1 years on the date of surgery. Specimens were supplied by the Department of Urology, (University Hospital Mannheim, Germany). The tissue was fixed in 10% buffered paraformaldehyde (Sigma Chemical, St Louis, USA) for 24 h and embedded in paraffin. Sections from each tissue block were stained with hematoxylin/eosin for tumor staging (in accordance with TNM classification 1997) and grading (Gleason method [12]). Out of the 56 total specimens, 26 were staged as T2, 26 as T3, and 4 as T4. The specimens were subdivided into high differentiation (Gleason sum score 2–6, 27 specimens), intermediate (score 7, 21 specimens) and poorly differentiated specimens (score 8–10; 8 specimens). The major tumor-containing block of each prostate cancer specimen was immunohistochemically evaluated.

Immunohistochemical staining and analysis

For immunohistochemical analysis, 3-µm serial sections were mounted onto slides (Superfrost/Plus; Menzel, Braunschweig, Germany) and dried at 37°C overnight. The sections were deparaffinized in Neoclear (Merck) and rehydrated in graded alcohol (100, 96, 80 and 70%) and then washed twice in PBS (phosphate buffered saline, pH 7.4; Sigma, Taufkirchen, Germany) for 5 min. Sections were stained using either the standard ABC-method (Vector Laboratories, Burlingame, USA) or DAKO EnVision + ® system (Glostrup, Denmark). The endogenous peroxidase activity was blocked by incubation with 1% hydrogene peroxide in methanol for 30 min and then washed twice in PBS. For antigen retrieval, the sections were power-cooked in citrate buffer (pH 6.0; DAKO) three times at 600 W in a microwave oven. Non-specific binding was blocked by incubation with either horse non-immune serum (staining for CD31 and VEGF; Vector Laboratories), goat non-immune serum (EGF staining, dilution 1:66 in PBS, DAKO) or rabbit serum (bFGF, Vector Laboratories) for 20 min. All primary and secondary immunoglobulins were diluted in PBS and incubated in a humid chamber.

CD31

Specific staining for endothelial cells was conducted by the use of a monoclonal mouse anti-human immunoglobulin, raised against the PECAM-1 molecule (DAKO). Slides were incubated overnight at 4°C (dilution 1:25). After two washes with PBS, the sections were incubated with a biotinylated horse anti-mouse antibody (AB) for 30 min at room temperature (RT) and then incubated with the ABC peroxidase complex (Staining Kit, Vector Laboratories). Diaminobenzidine (DAB, Vector Laboratories) served as a chromogen. Tissue sections were shortly counterstained with Mayers Hämalaun, rinsed in water, dehydrated in graded ethanol (70/80/96/100%), washed in Neoclear (2×2 min) and mounted (Neo-Mount, DAKO).

VEGF

The primary AB was monoclonal mouse anti-human VEGF(C-1) (Santa Cruz Biotechnology, Santa Cruz, USA) in a dilution of 1:500 used in an overnight staining procedure at 4°C. The remainder of the procedure was conducted as described above. VEGF(C-1) is directed against human VEGF, not detecting specific spliced isoforms.

EGF

Expression of EGF was visualized by the use of rabbit anti-human EGF immunoglobulin overnight at 4°C (dilution 1:25, Oncogene Research Products, San Diego, USA) and the EnVision + ® system. Peroxidase-conjugated secondary AB was incubated for 30 min at RT. After double washing with PBS, NovaRed (DAKO) was used as a chromogen. The remainder of the procedure was performed as described above.

bFGF

Expression was measured by the use of a polyclonal goat anti-human immunoglobulin (bFGF-2 (147)-G, Santa Cruz Biotechnology). Optimal dilution was determined to be 1:1000 in an overnight procedure at 4°C. Except for the use of a biotinylated rabbit anti-goat secondary IgG (Vector staining kit), the remainder of the procedure was conducted as described above.

The primary antibody was omitted for negative control in all stainings.

Quantification of VEGF, bFGF and EGF

Tumor sections stained for each of the three growth factors were analyzed semiquantitatively by the number of positive cancer/epithelial cells as well as the intensity of immunohistochemical staining (I) in a standard area (7.9 mm^2) of maximum expression within the carcinoma called the "hotspot" (II) within the entire carcinoma of the investigated section and (III) in surrounding non-tumorous tissue. Briefly, up to 5% positive tumor cells were considered (–), 6–49% as (+), 50–94% as (++) and 95% or more as (+++). Category (–) and (+) were rated as low expression, (++) and (+++) as high expression. In addition, the standard area (I, see above) was marked. The staining intensity was graded semiquantitatively as no immunoreactivity, weak reaction, strong reaction and very strong reaction and evaluated in each of the three above-mentioned regions.

Assessment of area with maximum MVD

The assessment of MVD was performed by light microscopy. The intratumoral area of maximum microvessel density was identified and marked (7.9 mm^2) as described earlier [13]. Any stained

endothelial cell or cell cluster separated from other microvessel structures was taken to be a countable microvessel. Notably, a lumen was not necessary for a structure to be counted as a microvessel.

Topographic relationship of maximum MVD and VEGF, bFGF and EGF

The topographic relationship between the intratumoral area of maximum microvessel density (see above) and the location of the highest expression of the different pro-angiogenic stimuli (area (I), see above) was investigated as previously described with slight modifications [13]. Briefly, CD31-stained sections and slides of one of the mitogenes were superposed and standard areas compared topographically by projection. The topographical relationship was classified into four categories: areas were identical (a), overlapping (b), had contact (c), or no topographical relationship existed (d).

Statistical analysis

All statistical analysis was performed with SAS for Windows (Version 8.02; SAS Institute, Cary, NC, USA). For statistical comparison of growth hormone expression in different areas of the section, the number of positive cells and the intensity of staining were ranked in four categories as described and the Sign test was used. A $p < 0.05$ was considered to be significant. For comparison of data on growth hormone expression and tumor staging/grading the Wilcoxon/Kruskal-Wallis rank sum test was used.

Results

VEGF

With one exception, positive staining in malignant and benign epithelial cells was observed in 53 VEGF-stained prostate cancer specimens. The expression of VEGF was assessed in three regions of the tissue specimen according to two criteria: number of positive cells and staining intensity. Examples of sections with a high/low number of VEGF-positive cells as well as strong or weak staining intensity are displayed in Fig. 1.

In standard "hotspot" areas of the tumor, 41 specimens (77.3%) displayed a high number of VEGF-positive cells (positive staining reached 95% or more cells in 20 of these cases). A significant difference between "hotspot" and evaluation of the entire investigated tumor was observed ($p < 0.0001$): within the entire tumor, 35 specimens (66%) displayed a high number of VEGF-positive cells, with only four entire tumor specimens showing 95% or more positive cells. In opposition to tumorous tissue, non-tumorous surrounding tissue showed a statistically significant lower number of positively stained epithelial cells ($p < 0.0001$): only 26% displayed a high number of stained cells. None of the benign regions displayed more than 95% VEGF-positive cells. The relevant data is summarized in Table 1.

The staining intensity was assessed as a secondary characteristic. In standard areas of tumor hotspots, 26.4% of specimens were rated as very strong, whereas in an overall tumor evaluation as well as in non-tumorous regions of the section, only two specimens (4.0%) were ranked equally. In malignant tissue, 30.1% (hotspot: 15.1%) of the investigated specimens showed no or weak staining intensity, whereas 46% of the non-tumorous tissue displayed no or weak staining intensity. No significant difference in number of positive cells and staining intensity between organ-confined and non-or-

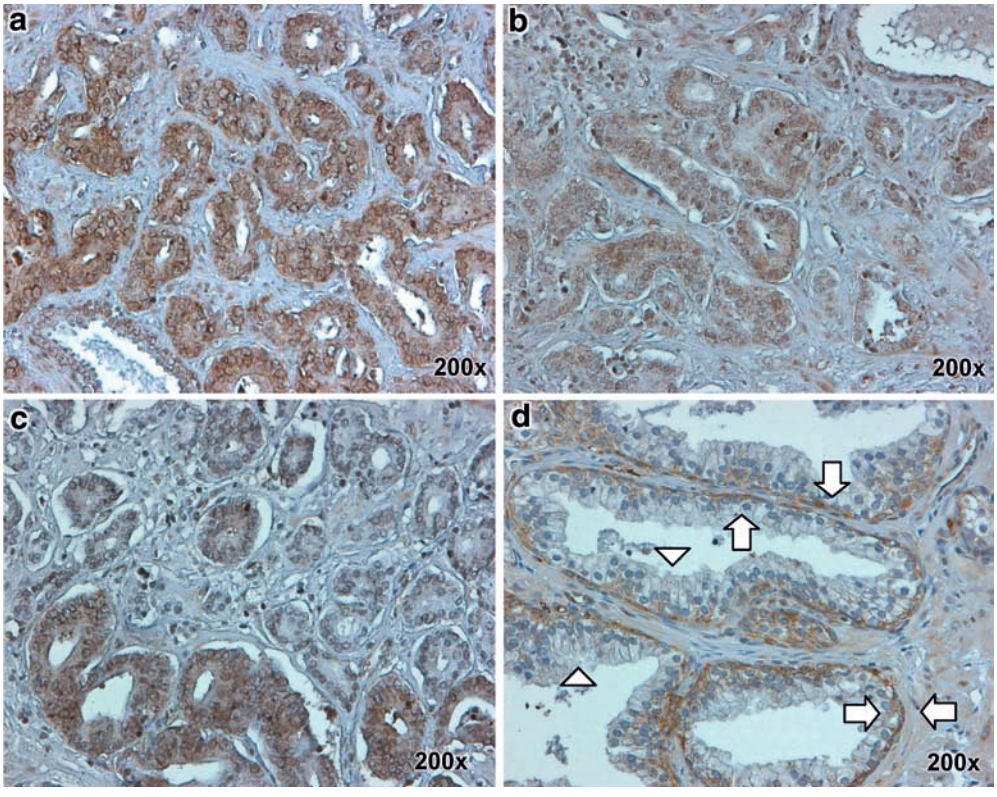

Fig. 1a–d Expression of VEGF in malignant and benign prostatic tissue (magnification 200×). Different VEGF staining intensities were observed: very strong (**a**) and weak staining intensity (**b**) in prostate cancer specimens. Number of VEGF-positive cells was variable (**a, c**). In most cases of benign tissue, VEGF expression was restricted to the basal cell compartment (**d,** *arrows*) with VEGF-negative luminal cells (*arrowheads*)

Table 1 Number of positive epithelial/cancer cells stained for the three growth hormones VEGF, EGF and bFGF. Staining was assessed in three regions of tissue specimens: in a standard area of tumor with maximum expression of the growth factor (“hotspot”), within the entire tumor and in non-tumorous tissue. Staining of 0–5% positive cells is considered as negative (−), 6–49% as (+), 50–94% as (++) and ≥95% as (+++)

VEGF	Hotspot	Tumor	Non-tumorous tissue
	n = 53	n = 53	n = 50
Low (−)/(+)	(22.7%), n = 22	(34.0%), n = 18	(74.0%), n = 37
High (++)/(+++)	(77.3%), n = 41	(66.0%), n = 35	(26.0%), n = 13
EGF	Hotspot	Tumor	Non-tumorous tissue
	n = 56	n = 56	n = 53
Low (−)/(+)	(35.7%), n = 20	(50.0%), n = 28	(86.7%), n = 46
High (++)/(+++)	(64.3%), n = 36	(50.0%), n = 28	(13.3%), n = 7
bFGF	Hotspot	Tumor	Non-tumorous tissue
	n = 48	n = 48	n = 46
Low (−)/(+)	(27.1%), n = 13	(39.6%), n = 19	(87.0%), n = 40
High (++)/(+++)	(72.9%), n = 35	(60.4%), n = 29	(13.0%), n = 6

gan confined tumors as well as high and low grade tumors was observed. Additionally, in most benign glands, the expression of VEGF was restricted to the basal cell compartment of the gland, whereas in malignant tissue, entire formations of malignant cells stained positive (see Fig. 1).

EGF

Following the same evaluation of 56 prostate cancer specimens stained for EGF, high expression of EGF was observed in 50.0% of the cancer specimens (within standard area of hotspot: 64.0%), whereas only 13.2% of non-tumorous tissue revealed a high number of positive cells. In contrast to VEGF staining, none of the cancer specimens and none of the non-tumorous regions showed more than 95% positive cells (hotspot: 16.1%). Comparison of tumor-hotspot and entire tumor as well as tumorous and non-tumorous tissue revealed a statistically significant difference in percentage of positive cells ($p < 0.0001$). This is summarized in Table 1.

Staining intensity differed within the tumor regions. As with VEGF, enhanced focal staining within the tumors was observed: 32.2% of hotspot regions were ranked as strong, but in an overall tumor assessment only 14.3% showed strong staining intensity. No difference was observed between tumorous and non-tumorous tissue. Representative EGF staining is depicted in Fig. 2. EGF staining intensity or number of EGF positive cells did not correlate with either tumor staging or Gleason grade of tumors.

bFGF

In contrast to both VEGF and EGF, staining intensity of bFGF revealed a significantly stronger expression in non-tumorous tissue compared with carcinoma. Even stronger and very strong stained cells were seen in the non-tumorous compartment of the section (63.0%) than in the region of tumor (8.5%; $p < 0.0001$) or even in the standard area of hotspot (16.7%).

In accordance with investigations on VEGF and EGF, the number of positive cells was enhanced in the tumorous tissue as compared with the non-tumorous tissue of the section: 60.4% of the tumor but only 13.0% of the non-tumorous tissue showed a high number of positive cells, $p = 0.02$. On the other hand, it was observed that, unlike VEGF and EGF, all of the investigated non-tumorous specimens stained positive for bFGF to some extent.

In accordance with data on VEGF and EGF, no enhancement of the bFGF staining intensity or increase in number of positive cells was observed in advanced tumors (non-organ confined tumors, high Gleason score).

Similar to VEGF and EGF staining, bFGF expression is stronger in basal layers of the benign glands (see Fig. 3).

Topographical relationship

It was possible to assess the topographical relationship of standard areas containing maximum vessel density and the three different proangiogenic stimuli in 51 cases (VEGF), 46 tumor specimens (EGF) and 44 specimens (bFGF). Topographic comparison of VEGF and MVD revealed an identical area of highest MVD and maximum VEGF expression in 12 cases (23.5%) and overlapping areas or areas in contact in 22 cases (43.1%), whereas 17 specimens (33.3%) showed no contact of standard areas at all. In contrast, maximum area of EGF was identical with maximum MVD in only 6.5% (three cases) and 15.9% (seven specimens) in the case of bFGF. Taking identical, overlapping or in contact areas together, VEGF displays a close topical relationship in 65% of investigated cases, EGF in 57% of tumor specimens and bFGF in 43%. The data is summarized in Table 2.

Discussion

Tumor growth and metastasis depend on the process of angiogenesis. This process is regulated by inhibiting and stimulating factors (e.g., VEGF, EGF and bFGF). The latter were investigated in this study.

The evaluation of human organ-specific angiogenesis is important for the development of therapeutic strategies, as animal tumor models or in vitro experimental set-ups do not accurately reflect the interactions between

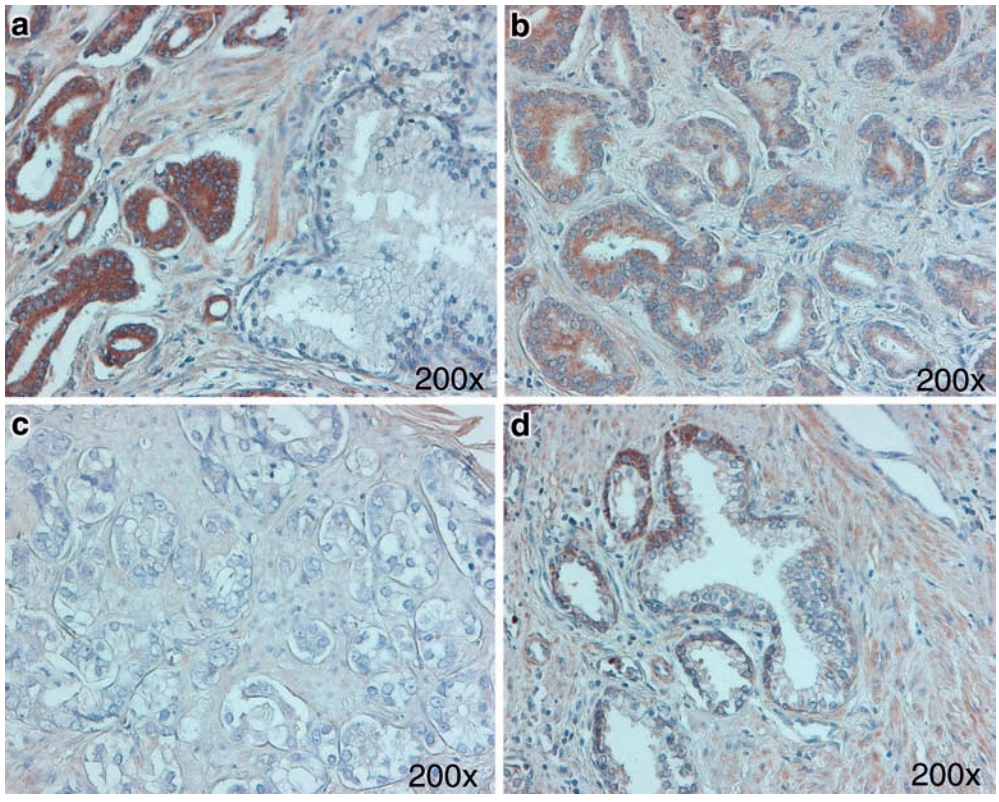

Fig. 2a–d Immunohistochemical staining for EGF in malignant and benign prostate tissue. Very strong staining (+ + +) of all malignant cells is shown in **a**, whereas benign aspects of the tissue remain unstained. Within the same section, a weak staining intensity was also observed (**b**), whereas no staining (−) was seen in some of the investigated malignant specimens (**c**). Benign tissue stained for EGF (**d**) but was mostly restricted to basal cell layers of the gland

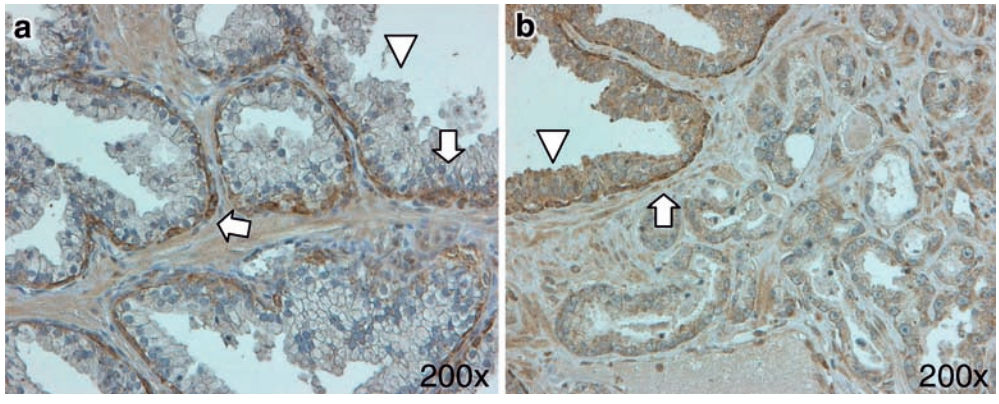

Fig. 3a, b Immunohistochemical staining of bFGF in prostatic tissue of benign (**a**) and malignant origin (**b**). In benign tissue, intense staining of the basal cell compartment is observed (*arrows*), whereas luminal cells show minor or no staining for bFGF (*arrowheads*). Prostate cancer shows minor staining intensity for bFGF compared with benign tissue (**b**)

the microenvironment of the organ and the investigated cancer [5]. The role of VEGF, EGF and bFGF in angiogenesis is not a matter of debate; several reports have been published on the expression of these growth factors by prostate cancer cells as well as their influence on endothelial proliferation and angiogenesis [14, 15, 16]. Recent immunohistochemical investigations on growth factor expression were restricted to the assessment of a single growth factor and do not allow comparative conclusions on pro-angiogenic stimuli in prostate cancer [14, 17, 18, 19, 20]. Comparison studies assessing different potential growth factors are still missing. Therefore, the role of the individual pro-angiogenic stimulus—in context of several known stimuli—remains unclear.

VEGF and its receptors are already a target for anti-angiogenic therapeutic strategies. In our study, the number of VEGF-secreting cells was statistically enhanced in tumorous tissue when compared with non-tumorous areas of the sections. These results concur with previous reports [19, 20]. In over 66% of investigated cases, more than 50% of neoplastic cells secreted VEGF. Remarkably, statistically relevant focal differences in VEGF expression within the tumor were observed: 20 investigated cases showed areas of more than 95% positive cells. The focally enhanced expression of VEGF seems to functionally influence angiogenesis. VEGF hotspots displayed the highest rate of topographical relationship to areas of strongest angiogenesis and can therefore be identified in the investigated specimens as the most prominent stimulus for angiogenesis in PCa. Previously, an increased staining intensity of VEGF in PCa compared with benign tissue was reported [19]. In the tissue sections included in this study, no

Table 2 Topographic relationship of maximum expression of the growth factors VEGF, EGF and bFGF and maximum microvessel density. Topographical relationship was subdivided into four categories: (a) 'hotspots' identical, (b) overlapping, (c) tangent and (d) no contact at all. Identical, overlapping or tangent 'hotspots' of CD31 and VEGF/EGF/bFGF sections are considered to be closely topographically related and expressed as percentage of investigated sections

	VEGF n = 51	EGF n = 46	bFGF n = 44
Identical	12 (66.7%)	3 (56.5%)	7 (43.2%)
Overlapping	14	13	6
Contact	8	10	6
No contact	17 (33.3%)	20 (43.5%)	25 (56.8%)

changes were observed in the staining intensity of VEGF, although the number of investigated sections was twice as high.

In the investigated sections, EGF was expressed by benign and malignant epithelial cells, which is in concordance with previous results [21]. Expression of EGF is increased in prostate cancer in terms of number of positive cells. Several previous reports proclaim an additional increase in staining intensity [17] not observed by us.

bFGF staining in malignant tissue displayed a significantly weaker staining intensity compared with the benign area of the prostate, although the number of positive cells is enhanced in PCa. These results somewhat contradict previously published results in which, in tissue extracts and cell supernatants, FGF expression (ELISA technique) was increased in PCa [15, 18], but no expression of FGF in benign epithelial or cancer cells was observed by immunohistochemistry [18]. One explanation for the different findings could be the variation of isoforms of FGF investigated [6] and the different antibodies used. On the other hand, Edgren et al. [22] confirmed our observation: serum analysis of bFGF in patients with PCa was no difference than that of healthy controls.

Conclusion

In conclusion, VEGF, EGF and bFGF were expressed in a large number of prostate specimens and could be related to angiogenesis in the tumor although the individual importance of the three different growth factors seems to vary. It is obvious that growth factors are not expressed in a unique pattern either within the tumor or in surrounding non-tumorous tissue—statistically relevant changes in malignant tissue were observed.

The topographic relationship of areas of maximum vessel density and the expression of the growth factors VEGF and EGF may further support the hypothesis that prostatic cancer cells act in a paracrine fashion to locally induce angiogenesis. Our data suggest that VEGF represents the strongest stimulus for endothelial cells within the carcinoma, displaying the highest percentage of a topographical relationship and maximum share of positive cancer cells within the tumor. Therefore, new therapeutic strategies taking VEGF and its receptors as a target seem to be a valuable treatment option for prostate cancer. EGF was identified as a second, strong promoter of angiogenesis and should be considered as an anti-angiogenic target for prostate cancer in the future. On the other hand, bFGF staining intensity was remarkably reduced in cancer and a topographical relation to maximum angiogenesis was seen in only a few of the investigated cases.

These findings support further investigations in the field of anti-angiogenic therapy, although it must be born in mind that some tumors apparently rely on different pro-angiogenic stimuli and mechanisms to induce angiogenesis.

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