Alteration of the Vascular Endothelial Growth Factor and Angiopoietins-1 and -2 Pathways in Transitional Cell Carcinomas of the Urinary Bladder Associated with Tumor Progression

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Abstract. Neoangiogenesis is assumed to play an important role in the progression, metastasis and prognosis of a wide variety of tumors. To get insights into the molecular-genetic pathways and the biological role of angiogenesis in urothelial carcinogenesis, we analyzed comparatively the expression of the mRNA of the vascular endothelial growth factor (VEGF) and of the angiopoietins-1 and -2 (Ang-1 and Ang-2) in 71 transitional cell carcinomas (TCC) of the urinary bladder in relation to the tumor grades and stages, and referring to epidemiological risk factors. Using real-time quantitative reverse transcription-polymerase chain reaction, low-stage superficial TCC expressed VEGF and Ang-2 mRNA at a significantly higher level than high-stage muscle invasive carcinomas, and low-grade TCC at an insignificantly higher level than high-grade tumors. The activity of both angiogenic factors was found to be significantly correlated. Conversely, Ang-1 mRNA was expressed at a 3-fold significantly lower level in low-grade, low-stage compared to high-grade, high-stage TCC. A significantly 3- and 2-fold, respectively, drop of the VEGF and Ang-2 mRNA expression in conjunction with a 2-fold significantly higher expression of Ang-1 mRNA in the group of grade 2 TCC when infiltrating the muscle layer may represent a crucial event during urothelial carcinogenesis, and possibly indicates an important step in promoting the conversion of bladder cancer from a low to a high malignancy in this subset of carcinomas. By immunohistochemistry, high-grade, high-stage carcinomas less frequently displayed areas with a strong reactivity for the VEGF protein (“hot spots”) than low-grade, low-stage TCC, paralleling the expression of the mRNA. The expression patterns observed are compatible with a reduced vascular destabilization and decreased formation of new blood vessels in advanced TCC, suggesting a balance between vessel regression and vascular growth, with a less pronounced vascular remodeling during late phases of urothelial carcinogenesis. Analyzing the effect of life-style bladder cancer risk factors, habitual smoking and coffee consumption was not observed to substantially alter the expression of the angiogenic mediators, except for weakly elevated levels of VEGF and Ang-2 mRNA in TCC of strong smokers and a borderline significantly decreased VEGF mRNA expression associated with heavy coffee consumption. Certain hazardous occupational exposures (polycyclic hydrocarbons, paints and lacquer, stone dust) may play a role in modulating tumor angiogenesis. The current data indicate that the signaling molecular-genetic pathways underlying vascular remodeling are involved in the progression of urinary bladder cancer to a more malignant and aggressive behaviour.

Angiogenesis is essential for tumor growth and is assumed to play a prominent role in the progression, metastasis and prognosis of a wide variety of tumors, including cancer of the urinary bladder (for review of the literature see 1-5). Physiological and pathological angiogenic activity is regulated by a complex concerted interaction and dynamic balance of proangiogenic and antiangiogenic mediators. Because of their specific effects on endothelial cells, vascular endothelial growth factor (VEGF) and the members of the angiopoietin family are of major interest among angiogenesis inducers. In urinary bladder cancer, increased microvessel density was
Figure 1. Real-time RT-PCR standard curves of β-actin, VEGF-A, Ang-1 and Ang-2 mRNA at serial dilutions (100, 10, 1, 0.1 and 0.01 attomol gene-specific PCR fragments) demonstrating high amplification efficiency.
associated with a poorer outcome and an increased risk of recurrences (6-9). The few molecular-genetic investigations available at present have yielded contradictory results. While, in some studies, a higher expression of VEGF mRNA was documented in low-stage superficial compared to high-stage muscle invasive transitional cell carcinomas (10-12), others reported the opposite (13, 14).

To obtain further insights into the signaling molecular pathways and biological role of bladder cancer angiogenesis, we analyzed the expression of VEGF at the mRNA and protein level in conjunction with the mRNA expression patterns of angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) using real-time quantitative reverse transcription-polymerase chain reaction. On the assumption that different carcinogenic agents may induce exposure-specific genomic instabilities (15-19), it was a further objective to examine whether the molecular-genetic findings can be related to life-style bladder cancer risk factors and hazardous occupational exposures.

Materials and Methods

Patients and sample collection. Transitional cell carcinomas of the urinary bladder were obtained from 71 patients who had undergone transurethral resection or, rarely, radical cystectomy. The cases were identical with those previously used for analysis of the tumor suppressor genes RB and p16 (20). Immediately after surgical removal, all tissue samples were initially stored in "RNAlater" (Ambion, Austin, USA) and subsequently snap-frozen at -70°C. For accurate histopathological diagnosis, additional tumor specimens were formalin–fixed and paraffin-embedded; section of 4 µm thickness were prepared and routinely stained with hematoxylin and eosin. The carcinomas were graded using the classification of the World Health Organisation (21) and staged according to the guide lines of the International Union Against Cancer (22). Non-neoplastic, normal appearing bladder mucosa was obtained from cystectomy specimens of 6 tumor patients as control. The investigation was performed according to the instructions of the local Ethics Committee and with the informed patient’s consent.

Epidemiological inquiry. In an attempt to identify patterns of altered gene expression potentially associated with known or suspected lifestyle and occupational risk factors of bladder cancer, the patients were interviewed at the hospital according to a standardized questionnaire that had previously been used in an epidemiological case-control study (23-25). Respondents were asked about their smoking habits including the average number of cigarettes/cigars or pipes smoked per day, years of consumption and date when they eventually stopped smoking. One cigar was defined as being equal to 7 and one pipe to 3 cigarettes. Current smokers were those who regularly had consumed at least 1 cigarette daily for at least 1 year before diagnosis of their bladder cancer. Current smokers were stratified into 4 categories: those smoking between 1 and 20 or more than 20 cigarettes per day, and those smoking for between 1 and 30 or longer than 30 years. Multiplication of the number of cigarettes consumed per day and the years of smoking yielded a so-called smoking index. Ex-smokers had stopped smoking for at least 10 years prior to cancer diagnosis. Subjects who had never consumed tobacco were classified as non-smokers. Patients were also asked to give their full occupational history. Detailed information was obtained on every employment held for at least 1 year; in addition, data were collected about occupational exposure to hazardous chemicals, dust or fumes lasting for 1 year or longer. Study participants subjected to several exposures are represented in more than one occupation category (see corresponding figures). The interview also included questions on coffee drinking, elucidating the number of cups consumed per day.

Immunohistochemistry. Sections of 4 µm thickness were prepared from the formalin-fixed, paraffin-embedded samples, deparaffinized in xylene, rehydrated in graded ethanol solutions and washed with distilled water. For detection of VEGF protein, the sections were pretreated by microwaves (700 W) for 5 minutes, each in citrate buffer (pH 6). The sections were then cooled down for 20 minutes, washed in distilled water and incubated with 3% hydrogen peroxide for 10 minutes at room temperature. Following washing in distilled water and Tris-buffed saline (TBS; pH 7.4; 0.05 M), the sections were covered with bovine serum albumin (10% in bi-distilled water) for 15 minutes at room temperature. The primary antibody (rabbit polyclonal anti-VEGF, Z-CVF3; Zymed Laboratories Inc.; San Francisco, USA) was applied at a dilution 1 : 50 in TBS at 4°C overnight. After rinsing with TBS, the sections were subjected to the Dako EnVision-System containing the secondary antibody (Chem Mate™ EnVision™ HRP, anti-mouse rabbit; Code-No. K 5007; Dako Diagnostika, Hamburg, Germany) for 30 minutes. Following washing with TBS, 3,3-diaminobenzene-tetrahydrochloride (DAB; Dako Diagnostika) was used as chromogen to visualize the sites of immunoprecipitation. The sections were finally washed in distilled water and counterstained with Mayer’s hemalaun. Transitional cell carcinomas were scored as strongly positive when they contained...
areas with an intense cytoplasmic staining, called "hot spots". For negative staining, the primary antibody was omitted.

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR).

RNA preparation. The snap-frozen surgical specimens were pulverized using a microdismembrator (B.Braun Biotech International, Melsungen, Germany). Smears were prepared from the pulverized material, stained with May-Grünwald-Giemsa and checked by light microscopy for sufficient tumor material (at least 70% tumor cells). Total RNA was extracted with Tri Reagent (Sigma-Aldrich Chemie, Taufkirchen, Germany) according to the recommendations of the manufacturer. The extracted RNA was purified using the Qiagen RNeasy Kit (Qiagen, Hilden, Germany). The integrity of the RNA was assessed by separating on a 1.5% agarose gel and staining with ethidium bromide (5 µl/40 ml agarose). The RNA was stored at -70°C until use.

cDNA synthesis. The 20-µl reaction mixture for reverse transcription contained 1 µg RNA, 200 units Superscript II RNase H-reverse transcriptase (Invitrogen Corporation Karlsruhe, Germany), 4 µl 5 x first-strand buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂], 2 µl DTT-solution (0.1 M), dNTP’s each at a concentration of 500 µM (Roche Molecular Biochemicals, Mannheim, Germany), 3 µg random primers (Invitrogen) and 40 units RNaseOUT recombinant ribonuclease inhibitor. The reaction mixture was incubated at 42°C for 50 minutes and subsequently inactivated by heating at 70°C for 15 minutes.

Standard curves. Standard curves were constructed from serial dilutions of gene-specific PCR fragments. These fragments had been amplified from human cDNA by PCR in a thermocycler (Biometra, Göttingen, Germany) and were purified from agarose gel using the QIAquick gel extraction Kit (Qiagen). The PCR reactions were performed in 50-µl volumes containing 2 µl cDNA, 2.5 units Taq DNA polymerase
Primers. RT-PCR was performed using the following primers: β-actin, sense 5'-CAT CAC CAT TGG CAA TGA GC-3', antisense 5'-TCG TAC TCC TGC TTG C-3' (product size 351 bp); VEGF, sense 5'-TAC TGC CAT CCA ATC GAC ACC-3', antisense 5'-AGC TCA TCT CTC CTA TGT GC-3' (product size 213 bp); Ang-1, sense 5'-CTG ACA GTA GAT GTT GAG ACC CA-3', anti-sense 5'-GTG TCC AAC TCT TTC ACC TG-3' (product size 199 bp); Ang-2, sense 5'-ATA AGC AGC ATC AGC CAA CC-3', anti-sense 5'-TGA GCC TTT CCA GTA GTA CC-3' (product size 203 bp). Each primer pair produced a PCR fragment spanning at least one exon boundary. The primers for VEGF-A were located on exons 1 and 4 and thus encompassed the coding region of all known splice variants of VEGF. All primers were obtained from MWG Biotechnology (Ebersberg, Germany).

PCR amplification. Quantitative real-time RT-PCR reactions were performed in a volume of 20 μl using an i-Cycler (Bio-RAD Laboratories, München, Germany). Each reaction mixture contained 50 ng cDNA, 1 unit HotStarTaq DNA polymerase (Qiagen), 2 μl 10 x reaction buffer [Tris-HCl (pH 8.7), KCl, (NH₄)₂SO₄, 15 mM MgCl₂], dNTP's (Roche Molecular Biochemicals) each at a concentration of 200 μM, 5 pmol sense - and 5 pmol antisense primer, SYBR green I (Molecular Probes Incorporation, Eugene, USA) in a final dilution 1 : 40,000, and fluorescein calibration dye (Bio-RAD Laboratories) diluted 1 : 10,000. The thermal cycling conditions for the i-Cycler consisted of an initial 15 minutes denaturation step at 95°C to activate the HotStarTaq-DNA polymerase. The amplification conditions for the various genes were as follows. β-actin and VEGF-A: denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, extension at 72°C for 45 seconds; angiopoietins-1 and-2: denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, extension at 72°C for 45 seconds; angiopoietins-1 and-2: denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, extension at 72°C for 45 seconds. For amplification, 50 cycles were performed. Examples of the amplified PCR products are demonstrated in Figure 2.

Expression of the mRNA was determined simultaneously for all tumor samples and the normal probes in two separate runs. The mean expression value of the threshold cycles from the two runs was standardized to an external gene-specific standard curve. The
calculated values were divided by the expression value of a housekeeping ß-actin and finally depicted as the ß-actin/mRNA ratio.

Statistical analysis. For statistical analysis, the non-parametric two-sided Wilcoxon rank sum test for paired group comparisons was applied. The data are presented as box plots demonstrating the 25th and 75th percentiles, the median value and the minimum and maximum values. The relationship between the mRNA expression of the angiogenic factors was analyzed by Spearman's rank correlation test. A \( p \)-value of at least 0.05 was considered to be statistically significant.

Results

Histopathological findings. Among the 71 urothelial carcinomas of the urinary bladder studied, there were 52 TCC with a papillary pattern of growth of various grades and stages (26 grade 1, 22 grade 2 and 4 grade 3; 42 pTa, 4 pT1, 5 pT2 and 1 pT3), and 19 non-papillary (solid) carcinomas (5 grade 2 and 14 grade 3; 17 pT2 and 2 pT3). 10 of the papillary and 3 of the non-papillary cancers represented recurrent tumors. The age of the patients ranged from 35 to 92 years (mean age 73 years). There were fifty-four men and 17 women.

Expression of vascular endothelial growth factor mRNA. The expression of VEGF mRNA was twice as high in transitional cell carcinomas, including all grades and stages (median 0.105; \( N = 71 \)), than in the normal appearing, non-neoplastic mucosa (median 0.048; \( N = 5 \)) of the bladder. Low-grade TCC (grade 1 and 2) expressed VEGF at a higher level (medians 0.105 and 0.112, respectively) than high-grade (grade 3) carcinomas (median 0.077), but the differences lacked statistical significance (Figure 3). A
A significant relationship \((p=0.02)\) was found between tumor stages and expression (Figure 3), low-stage superficial (pTa and pT1) TCC revealing an approximately 2-fold elevated expression (median 0.116) compared to high-stage muscle invasive (pT2 and pT3) carcinomas (median 0.066). Low-grade, low-stage TCC combined (grades 1 and 2, pTa and pT1) expressed the VEGF mRNA at an insignificantly \((p=0.11)\) higher level (median 0.124) than high-grade, high-stage (grade 3, pT2 and pT3) cancers (median 0.077). Among the group of TCC grade 2, those with only a superficial pattern of growth exhibited an approximately 3-fold significantly \((p=0.02)\) higher expression (median 0.141) referred to those infiltrating the muscle layer (median 0.050). Primary (median 0.112; N=57) and recurrent (median 0.086; N=13) TCC, including all grades and stages, did not differ significantly in their VEGF activity \((p=0.67)\).

**Immunohistochemical expression of VEGF protein.**

Immunohistochemical staining for VEGF protein was heterogeneous within the same carcinoma and proved to be weakly or moderately positive in most parts of the tumors. It was impossible to classify the intensity of reactivity into various grades or to establish the percentage of positive and negative cells, as practiced by others (14, 26, 27). Our evaluation was based on the presence of "hot spots", defined as easily recognizable circumscribed areas consisting of tumor cells with a strong intracytoplasmic staining (Figure 4). Of the 70 carcinomas examined, 53 (76%) contained such "hot spots", the occurrence of which showed an association with the grades and stages (Table I), low-grade, low-stage (G1, pTa/pT1) cancers more frequently displaying "hot spots" (83%) compared to high-grade, high-stage carcinomas (60%). The expression of VEGF mRNA was nearly twice as high (median 0.045) in carcinomas with "hot spots" than in those without (median 0.025; \(p=0.22\)).
Expression of angiopoietin-1 mRNA. Normal looking, non-neoplastic bladder mucosa expressed Ang-1 mRNA at a nearly 7-fold higher level (median 0.0060; N=6) than transitional cell carcinomas (median 0.0009; N=71), which were characterized by a gradual increase in the expression levels with increasing grades (Figure 5). TCC grade 3 exhibited a significantly (p<0.001) elevated expression (median 0.0019) compared to grade 1 carcinomas (median 0.0005) and a borderline significantly (p=0.06) higher expression level than grade 2 carcinomas (median 0.0008). A significant association was observed for the tumor stages (Figure 5): the expression was approximately 3-fold higher in high-stage muscle invasive (median 0.0060) than in low-stage superficial (median 0.0006) TCC (p=0.003). High-grade, high-stage carcinomas combined expressed the mRNA of Ang-1 significantly (p=0.001) at a 3-fold higher level (median 0.0020) than low-grade, low-stage tumors (median 0.0006). Among the TCC grade 2, those with infiltration of the muscle layer revealed a significantly (p=0.03) 2-fold higher expression (median 0.0014) compared to those with a superficial pattern of growth (median 0.0007). The Ang-1 activity did not differ substantially between primary (median 0.0007; N=13) and recurrent (median 0.0010; N=57) tumors.

Expression of angiopoietin-2 mRNA. Expression of Ang-2 mRNA was slightly higher in transitional cell carcinomas (median 0.0141; N=71) than in non-neoplastic bladder mucosa (median 0.0083; N=6). The expression level was found to be higher in grade 1 (median 0.0147) and grade 2 (median 0.0162) compared to grade 3 (median 0.0106) carcinomas, but the differences were not statistically significant (Figure 6). Low-stage superficial TCC expressed Ang-2 mRNA at a significantly (p=0.02) higher level (median 0.0163) than high-stage muscle invasive cancers (median 0.0103; Figure 6). Low-grade, low-stage carcinomas combined showed an elevated expression (median 0.0169) relative to high-grade, high-stage TCC (median 0.0096) with a borderline significance (p=0.07). A statistical significance (p=0.02) was evident for the 2-fold higher expression of grade 2 superficial (median 0.0242) compared to grade 2 muscle invasive (median 0.0111) carcinomas. The considerable differences between the expression levels of the individual tumors may reflect their cellular and molecular genetic heterogeneity, independent of their grades and stages. Recurrent (median 0.0134; N=13) and primary (median 0.0143; N=57) tumors did not differ in their Ang-2 gene activity.

Correlation analysis of mRNA expression of VEGF, Ang-1 and Ang-2. A highly significant correlation was observed between the expression of VEGF and Ang-2 mRNA (correlation coefficient r 0.62; p=0.0001) in TCC, including all grades and stages, while no correlation existed between the expression of VEGF and Ang-1 (r 0.12; p=0.31), or between the expression of Ang-1 and Ang-2 mRNA (r 0.17; p=0.15). Increasing tumor grades showed a significant increasing correlation between the expression of VEGF and Ang-2 genes was significantly associated in high-stage muscle invasive (r 0.83; p=0.0001) and, to a lesser extent, in low-stage superficial (r 0.40; p=0.06) carcinomas. Expression of Ang-1 and Ang-2 mRNA proved to be correlated only in grade 3 TCC (r 0.46; p=0.05) and in superficial cancers (r 0.39; p=0.008).

Expression of the angiogenic mRNA in relation to lifestyle and occupational risk factors. Analyzing the effect of lifestyle bladder cancer risk factors, carcinomas of habitual smokers including all categories of consumption and referred to the smoking index demonstrated an expression of VEGF, Ang-1 and Ang-2 mRNA comparable to that of non-smokers and ex-smokers (Table II). Stratifying for the various consumption categories, smoking of more than 20 cigarettes daily (N=7) was associated with an insignificantly elevated expression level of VEGF (median 0.141; p=0.18) and Ang-2 mRNA (median 0.0242; p=0.11) relative to smokers (N=23) of between 1 and 20 cigarettes (medians 0.098 and 0.0141, respectively). A time relationship could not be observed (data not shown). Coffee consumption was not found to substantially affect the expression of the angiogenic mediators, although the TCC of drinkers of 5
Physiological vasculogenesis and pathological angiogenesis are regulated by a complex interaction of pro-angiogenic mediators initiating vascular growth, such as the acidic and basic fibroblast growth factors, transforming growth factors alpha and beta and the platelet-derived endothelial growth factor and anti-angiogenic factors inhibiting formation of new blood vessels as, for example, angiostatin, thrombospondin, laminin peptides and tissue metalloproteinas inhibitors (for review of the literature see 1-3, 5). Tumor neoangiogenesis reflects a shift in the balance between angiogenic inducers and inhibitors, resulting in an increased angiogenic and a reduced angiostatic activity. Among the angiogenesis inducers, the members of the VEGF and angiopoietin family play a crucial role, since they act specifically on the endothelium by binding to receptors present on these cells. Tumor-associated angiogenesis depends on a cascade of sequential cooperative and coordinated interactions of the angiopoietins and VEGF, the molecular-genetic pathways of which remain to be further elucidated (1, 28-35).

This is the first investigation analyzing the expression of VEGF in conjunction with Ang-1 and Ang-2 mRNA in transitional cell carcinomas of the urinary bladder using real-time quantitative reverse transcription-polymerase chain reaction. VEGF stimulates proliferation of endothelial cells resulting in the formation of new blood vessels by sprouting from preexisting destabilized microvasculature (4, 29-31, 33-35). VEGF mRNA was found to be expressed at an approximately 2-fold significantly higher level in superficial low-stage (pTa and pT1) than in advanced muscle invasive high-stage (pT2 and pT3) TCC, suggesting activity of the VEGF gene to be associated with the tumor stages and indicating up-regulation during early, and down-regulation at late phases of urothelial carcinogenesis. The expression pattern is compatible with the well known fact that the stromal stalk is compatible with the well known fact that the stromal stalk representing a crucial event during urothelial carcinogenesis and possibly indicates conversion of carcinomas with a primarily low to those with a high malignant potential. Our results are in accordance with those of Crew and coworkers who documented a 3-to 4-fold higher expression of VEGF mRNA in superficial than in muscle invasive TCC using the ribonuclease protection assay (10-12). By contrast, the VEGF mRNA transcript was shown to be overexpressed in "invasive" as compared to "superficial" TCC by Northern blot hybridization – in muscle invasive relative to superficial non-invasive bladder carcinomas (13).

The protein expression of VEGF, assessed by immunohistochemistry, was associated with the tumor grades and stages and thus with the expression of mRNA,
low-grade, low-stage TCC displaying more frequently "hot spots" (consisting of tumor cells with a strong immunoreactivity) than high-grade, high-stage carcinomas. Similarly, Campbell and coworkers (26) reported a higher incidence of superficial low-grade carcinomas with a strong immunohistochemical positivity compared to muscle invasive high-grade tumors, some of which (2 out of 10 cases) even lacked detectable positive staining. Others observed a homogeneous immunoreactivity for VEGF and were unable to identify "hot spots" (14). Quantitative immunohistochemical determination of microvessel density using the antibody CD34 and factor VIII for staining endothelial cells did not provide any association with the grades and stages of TCC, but counts were restricted to selected areas containing the highest number of vessels (6, 9). However, a high microvessel density was found to be linked with an increased risk of recurrences, an increased incidence of lymph node metastases and a decreased overall survival, suggesting angiogenesis as an independent prognostic indicator (6-9). In another study a correlation with the patient outcome, recurrences and tumor progression was not observed (36). The findings of other authors who reported an increased recurrence rate of superficial (12, 37) and muscle invasive TCC (8) when expressing VEGF mRNA and urinary VEGF protein at elevated levels, suggesting VEGF gene activity as a predictor of disease recurrence, could not be substantiated in the current study.

The higher expression of VEGF mRNA in low-stage superficial TCC than in high-stage muscle invasive carcinomas coincided with an overexpression of Ang-2 mRNA. The close relationship between VEGF and Ang-2 gene activity is evidenced by the high correlation coefficients between the expression levels with respect to the tumor grades and stages, indicating a cooperative interaction of the two genes. Ang-2 products are released by endothelial and cancer cells as well (38, 39) and signal through the endothelial cell-specific Tie-2 receptor tyrosine kinase (29-31, 40). As a functional antagonist of Ang-1 that also binds to the Tie-2 receptor, Ang-2 protein primarily causes destabilization and disruption of preexisting microvessels and plays a key role in promoting angiogenic remodeling. A recent study demonstrated Ang-2 to exert a destabilizing vascular effect in the absence of and a stimulating effect on blood vessel formation in the presence of VEGF gene activity (32). The observed decreased expression of Ang-2 and VEGF mRNA in advanced high-stage TCC may point to a reduced vascular destabilization and a concomitant diminished recruitment of new blood vessels, suggesting a balance between vessel regression and vascular growth with a less pronounced vascular remodeling during late stages of urothelial carcinogenesis. The 2-fold significantly reduced expression of Ang-2 mRNA in muscle invasive compared to superficial TCC grade 2 in association with a 3-fold drop of VEGF and a 2-fold increase of Ang-1 mRNA expression support the concept that the shift in the expression patterns may indicate an important step for developing a more malignant potential in this subset of bladder tumors. The mechanisms underlying down-regulation of VEGF and Ang-2 gene activity in high-stage urothelial carcinomas are not clear. While there is a growing body of evidence that transcription of the VEGF and Ang-2 genes is activated by hypoxia, particularly by the oxygen-regulated factor HIF-1 (28, 40-44), the mediators responsible for blocking the angiogenesis inducers are unknown. The reduced angiogenesis at late stages of urothelial carcinogenesis results in impairment of the blood and oxygen supply leading to hypoxic necroses, frequently occurring in less differentiated and fast growing advanced muscle invasive, but never in well-vascularized superficial TCC. Our data are in favor of the hypothesis that hypoxia must be ascribed to the high malignancy of advanced bladder cancer and does not, vice versa, represent the causative agent for developing a more malignant tumor phenotype.

Stabilization of the angiogenic response at the late stages of bladder cancer development is further supported by a 3-fold significant increase of the Ang-1 mRNA in high-grade, high-stage compared to low-grade, low-stage transitional cell carcinomas. Ang-1 protein has been repeatedly documented to protect preexisting quiescent mature vasculature and is necessary for the maturation and stabilization of newly-formed immature blood vessels including the formation of a basal membrane and of pericytes, finally resulting in the development of a network of functional microvasculature (for review of the literature see 4, 29-31, 33-35).

In a first exploratory approach to analyze the significance of etiological life-style factors, habitual smoking as the most important bladder cancer risk factor was not identified to substantially affect the activity of the angiogenic mediators. There was only a weak trend for a dose-response relationship, suggested by slightly increased expression levels of VEGF and Ang-2 mRNA in carcinomas of patients who smoked more than 20 cigarettes daily compared to smokers of between 1-20 cigarettes. Similarly, coffee consumption as a further known risk factor (for review of the literature see 25) could not be documented to substantially alter the angiogenic process, except for a borderline significantly reduced expression of VEGF mRNA. Among the various exposures, only contact with polycyclic hydrocarbons, stone dust, paints and lacquer – known to be correlated with an increased risk of bladder cancer in several epidemiological case-control studies (for review of the literature see 23, 25) – proved to be associated with an elevated mRNA expression of the angiogenic
mediators. Although the increase of the expression levels did not reach statistical significance, possibly because of the relatively small sample size, our exploratory findings propose certain hazardous occupational exposures to affect the angiogenesis of urothelial carcinomas. Further research is required to confirm and extend the current data for definite conclusions to be drawn.

In conclusion, the present molecular-genetic investigation documented angiogenesis as playing an essential role during the progression of urothelial carcinogenesis. High-grade, high-stage transitional cell carcinomas expressed the mRNA of VEGF and Ang-2 at a lower while – conversely – the Ang-1 mRNA at a higher level compared to low-grade, low-stage carcinomas. The fundamental shift in the expression of VEGF, Ang-1 and Ang-2 mRNA observed in the group of grade 2 TCC when invading the muscle layer may represent a critical event during urothelial carcinogenesis and possibly signals a switch from a low to a high malignancy in this subset of cancers. Analyzing the effect of lifestyle bladder cancer risk factors, smoking and coffee drinking were not identified as substantially modulating the activity of the angiogenic inducers, although strong smoking was found to be associated with increased levels of VEGF and Ang-2 mRNA, and heavy consumption of coffee with a decreased expression of VEGF mRNA. Certain occupational exposures were linked with an elevated mRNA expression of all three angiogenic factors. The current approach highlights the complexity of angiogenesis in transitional cell carcinomas of the urinary bladder depending on the various phases of tumor development.

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