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Advances in Brief

The Angiogenic Factor Midkine Is Expressed in Bladder Cancer, and Overexpression Correlates with a Poor Outcome in Patients with Invasive Cancers[1]

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Abstract

Midkine (MK) is a member of a family of heparin-binding growth factors, which are reported to be angiogenic. We have investigated by RNase protection analysis the expression of MK in 47 primary bladder tumors and 7 normal bladder samples.

MK mRNA transcripts were detectable in 46 (98%) of 47 of the tumors and in 5 (70%) of 7 of the normal bladder samples. However, median MK expression was 4-fold higher in tumors than in the normal bladder ($P < 0.004$). In eight tumors (17%), MK expression was elevated more than 10-fold compared with the median value of the normal bladder specimens. There was no statistically significant difference in expression between superficial and invasive tumors ($P < 0.50$).

Seven (32%) of 22 patients with invasive cancers are alive at 1 year with no evidence of recurrence; in 5 (70%) of these patients, MK expression in the tumor was within the normal range at the time of presentation. By contrast, in only 2 (13%) of 15 patients who died or whose tumors recurred or progressed was MK expression in the normal range ($P < 0.01$). Overall, median MK expression in invasive tumors that caused death, progressed, or recurred within 12 months was 3-fold higher than that found in the tumors of those patients who were clear of disease at 12 months ($P < 0.04$).

Thus, overexpression of MK is associated with the development of bladder cancer and in invasive cancers predicts a poor clinical outcome in the short term.

Introduction

MK[3] is one of a family of recently cloned heparin-binding, neurotrophic factors, which are developmentally regulated and which may have an important role in angiogenesis and neoplasia (1–4).[4] The family includes MK, pleiotrophin, and the avian midkine homologue RI-HB.

MK was originally discovered in 1988 (1) as an embryonic retinoic acid-inducible gene, and in humans the gene is located on chromosome 11q11.2. MK protein is a highly basic secreted growth factor with a M_r of around 14,000. It is highly conserved across species and is produced by endothelial cells, fetal astrocytes, and proximal renal tubular cells (5). MK is strongly expressed in the developing mouse fetus, and the available evidence points to it playing a role in nervous system development, angiogenesis, and epithelial-mesenchymal interactions (1, 2, 6).[4] *In vitro* studies of the activity of MK have been somewhat conflicting, but MK appears to have both differentiation and mitogenic activity on neuronal cells, endothelial cells, and fibroblasts (6). Furthermore, MK has recently been shown to be angiogenic in a number of *in vitro* and *in vivo* assays.[4] A role for midkine in tumor angiogenesis is suggested by the observation that transfection of the breast carcinoma line, MCF-7, with midkine accelerates tumor growth, and increases tumor vascularity after implantation of the cells in nude mice.[4]

In normal adult tissues, MK expression is highly restricted. It is detectable in the kidney, lung, and thyroid but is only strongly expressed in the mucosa of the small intestine (3). This restricted pattern of expression in normal tissues makes expression in pathological states more likely to be of significance.

MK has been identified in several pathological tissues, including the neural plaques that characterize Alzheimer's disease (7), and some tumor types, including Wilm's tumor (3), neuroblastoma (8), breast cancer (9), and gastrointestinal cancers (3). In the series of Tsutsui *et al.* (3) MK was identified in all Wilm's tumors studied and in the majority of hepatocellular, stomach, and pancreatic cancers. In neuroblastomas, MK and pleiotrophin appear to be differentially expressed: MK is detected in nearly all primary neuroblastomas, whereas pleiotrophin is highly expressed only in neuroblastomas with a more favorable prognosis (8). Whether this pattern of expression is conserved in other tumor types is not known.

Angiogenesis is a prerequisite for tumor growth (10), and angiogenic activity, as determined by vascular density, has been shown to correlate with a higher incidence of lymph node metastases and a worse prognosis in numerous tumors, including bladder cancer (11). The degree of neovascularization appears to be determined by the competing actions of molecules that stimulate and those that inhibit angiogenesis. Many of these angiogenic factors have now been described, but their relative importance in determining tumor angiogenesis and growth in any given tumor type, including bladder, remains to be elucidated (10). We have recently described differential expression VEGF and PDECGF in different stages of bladder cancer and have demonstrated a close relationship between the recurrence of superficial tumors and overexpression of VEGF (12). However, it seems likely that other factors will also contribute significantly to the angiogenic phenotype in bladder cancer and, in so doing, will prove to be molecular determinants of recurrence and stage progression.

New approaches to the treatment of bladder cancer are urgently needed. Superficial tumors (tumors not invading detrusor muscle) have a recurrence rate of 40–70% despite treatment with surgery and/or intravesical chemotherapy, and only around 40% of patients with muscle-invasive cancers survive 5 years, however treated (13). Angiogenesis, and the molecules that control it, are of particular interest in this regard because of the highly selective target that they

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[3] The abbreviations used are: MK, midkine; VEGF, vascular endothelial growth factor; PDECGF, platelet-derived endothelial cell growth factor; FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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potentially present for antitumor therapy. In the normal adult, active angiogenesis takes place only in wounds, during the cyclical changes of the female reproductive tract, and during placentation.

The association of MK expression with the development of many tumor types and its known angiogenic activity led us to explore its role in bladder cancer.

Materials and Methods

Preparation of RNA. RNA was prepared from 47 primary bladder tumors and 7 normal bladder samples using the method of Chomczynski and Sacchi (14). The tumors were all obtained by transurethral resection of newly diagnosed bladder tumors. Tumors were staged by a combination of histological examination of tissue obtained at the time of transurethral resection and computed tomographic scanning of the pelvic organs. The normal bladder specimens came from patients with no evidence of urological disease. Patients were followed-up with regular cystoscopic examinations. Maximum follow-up is 2 years, and minimum follow-up is 9 months.

RNA was also prepared from three bladder cancer cell lines, 253J, T24, and RT4. The 253J and T24 lines act as paradigms of poorly differentiated tumors, whereas RT4 acts as a paradigm of a well-differentiated tumor. RNA was prepared from fresh human placenta and was used as a positive control on all gels.

Construction of Plasmids to Generate Probes for RNase Protection Analysis. A 486-bp fragment of human MK cDNA was cloned into the *Eco*R1 site of plasmid pBluescipt KS^+. The plasmid was linearized with *Eco*RV and an antisense fragment generated with T7 RNA polymerase.

Ribonuclease Protection Analysis. Antisense probes, labeled with [α-^{32}P]dCTP, were hybridized to 10 μg total cellular RNA, and the free unhybridized probe was removed by digestion with RNases A and T1. Protected fragments were analyzed by electrophoresis in 6% polyacrylamide-urea sequencing gels, followed by autoradiography. In each hybridization, and antisense transcript, corresponding to GAPDH transcribed from a construct, was included as an internal control. mRNA abundance was quantified by scanning laser densitometry (Bio Image analyzer; Millipore), and signals were standardized to the GAPDH control to provide a measure of expression. For each tumor, the relative signal was calculated by ascribing an arbitrary value of 100 to the signal from a positive control, which was loaded onto each gel to allow for cross-gel comparisons.

Statistical Analysis. The levels of expression of MK in normal bladder tissue and tumors were compared using the Mann-Whitney *U* test (two tailed). The clinical outcomes in patients with invasive cancers were compared using Fisher's exact test.

Results

Twenty-five of the tumors were superficial (pT_a and pT_1), and 22 were invasive (pT_2–pT_4). Of the superficial tumors, 4 were pT_a lesions (not invading lamina propria), and 21 were pT_1 lesions (invades lamina propria but not detrusor muscle). Three of the invasive tumors were predominantly squamous cell tumors, with all other tumors being transitional cell in origin. All of the pT_a tumors had papillary morphology; 16 of the pT_1 tumors were papillary, 2 were solid, and 3 were mixed. Three of the invasive cancers had papillary morphology, and 19 were solid.

Representative protection assays for MK are shown in Fig. 1. Midkine mRNA transcripts were detectable in 46 (98%) of 47 of the tumors and in 5 (70%) of 7 of the normal bladder samples. The range of MK expression across the tumors and the normal bladder specimens is shown in Fig. 2.

Median MK expression was 4-fold higher in tumors than in normal bladder tissue ($P < 0.004$). There was no statistically significant difference in expression between superficial and invasive tumors ($P < 0.50$). In 30 (63%) of 47 tumors, MK expression was higher than in any of the normal bladder specimens, and in 8 (17%) of the tumors, MK expression was elevated 10-fold compared with the median value of the normal bladder specimens.

The clinical fate of the 22 patients with invasive cancers is shown in Table 1. In five (70%) of the seven patients who are alive at 1 year, MK expression in the tumors was within the normal range (<39 densitometry units) at the time of presentation. By contrast, in only 2 (13%) of 15 patients who died or whose tumors recurred or progressed was MK expression in the normal range ($P < 0.01$). Overall, median MK expression in invasive tumors that caused death, progressed, or recurred within 12 months was 3-fold higher than that found in the tumors of those patients who were clear of disease at 12 months (Fig. 3). $P < 0.04$).

Eleven (53%) of the 21 patients with pT_1 tumors (not invading detrusor muscle but penetrating lamina propria) developed recurrences at 3 months, whereas 10 remained clear of disease. There was no statistically significant difference in median MK expression between pT_1 tumors that subsequently recurred at 3 months compared with those in which no recurrence was apparent ($P < 0.76$).

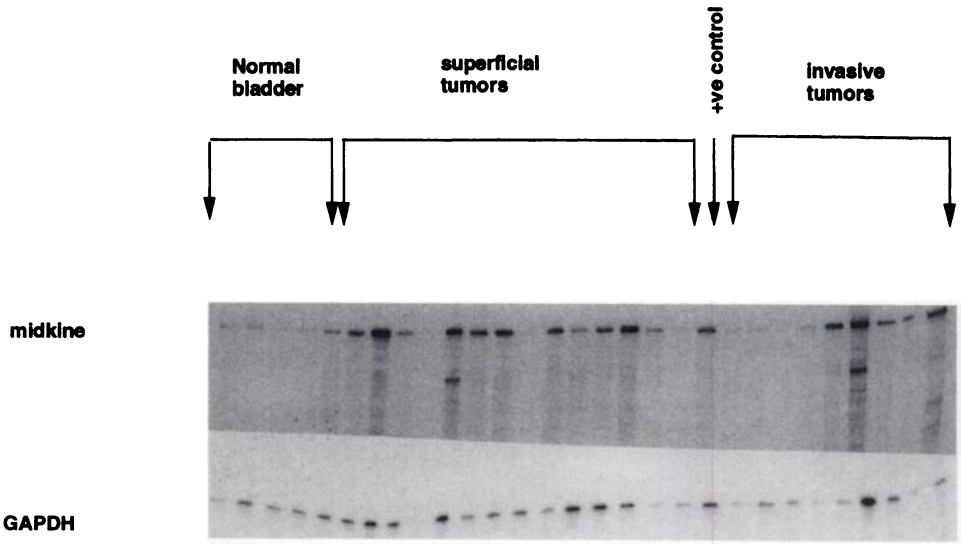

Fig. 1. Representative radiograph of ribonuclease protection assay of MK expression in bladder cancer.

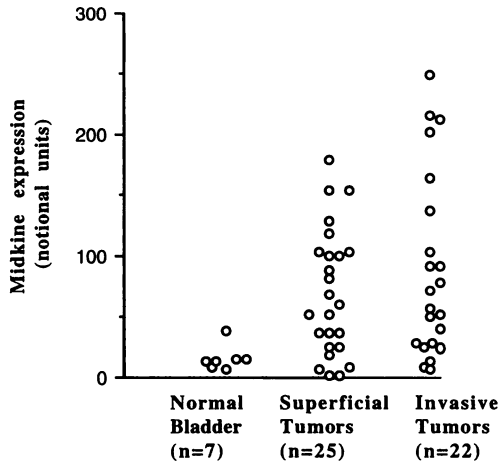

Fig. 2. MK expression in all samples. mRNA abundance was quantified by scanning laser densitometry (Bio Image analyzer; Millipore), and signals were standardized to the GAPDH control to provide a measure of expression. For each tumor, the relative signal was calculated by ascribing an arbitrary value of 100 to the signal from a positive control, which was loaded onto each gel to allow for cross-gel comparisons.

Discussion

Multiple peptide growth factors have been implicated in the development and vascularization of bladder cancers, including epidermal growth factor (13), acidic (15) and basic (16) fibroblast growth factors, VEGF, and PDECGF (12). The finding in this study that MK is expressed in 46 of 47 bladder cancers suggests that it may also play a significant role, particularly because in 63% of the cancers, MK expression was higher than in any of the normal bladder controls, and in 17% of cancers, expression was 10-fold higher than the median value for the controls.

Whether MK has a direct mitogenic effect on the epithelial component of the bladder cancers, or rather, produces an effect through modulation of stromal tissue or angiogenesis is not clear. The key physiological roles of MK remain to be elucidated, but the highly restricted pattern of expression of MK during development suggests that it plays a significant role in cell growth and differentiation as well as organogenesis. A role for MK in angiogenesis is suggested by reports that it is a mitogen for endothelial cells and that it stimulates angiogenesis in a number of *in vitro* and *in vivo* assays.[4] Furthermore, transfection of the breast carcinoma line MCF-7 with MK accelerates tumor growth and increases tumor vascularity after implantation of the cells in nude mice.[4] It is, therefore, possible that MK plays a central role in bladder cancer angiogenesis.

Angiogenic activity appears to be an important determinant of the behavior of bladder cancers, because vascular density has been shown in two studies to be an independent predictor of outcome in patients with invasive bladder cancer (11), and overexpression of VEGF correlates with recurrence of the pT_1 subset of superficial tumors (12). The precise identity of the angiogenic factors responsible for increased vascular density in some invasive tumors is not yet clear, but PDECGF (12), basic FGF (16), and acidic FGF (15) all play a role. Our previous finding of differential expression of VEGF and PDECGF in superficial and invasive bladder cancers led us to propose that there might be two angiogenic pathways in bladder cancer (12), in keeping with different genetic pathways (17). The results presented

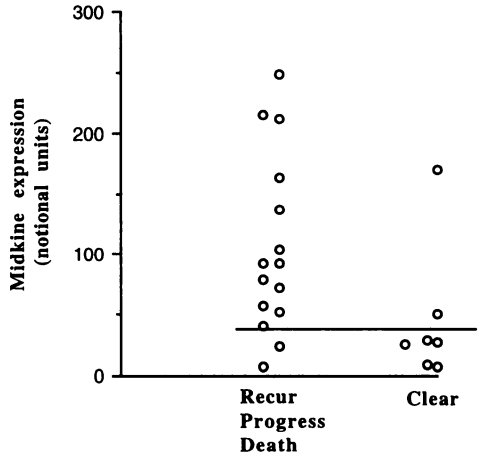

Fig. 3. MK expression in invasive cancers. *Left column*, range of MK expression in tumors that recurred, progressed, or caused the death of the patient within 1 year. *Right column*, range of MK expression in tumors that showed no signs of recurrence or progression at 1 year. *Horizontal line*, upper limit of MK expression seen in normal bladder specimens.

Table 1 *MK expression in invasive cancers: patient death, tumor pathology, adjuvant treatment, and clinical outcome in 22 patients*

Seven patients alive and free of disease at 12 months. In the tumors of five of these patients, MK expression was within the normal range (<39 notional units).

Patient details					
Gender[a]	Age (yr)	MK value	Stage and grade	Adjuvant Treatment	Outcome
M	75	248	T_3G_3	Partial cystectomy	Death <9/12
F	86	215	T_4G_3	Palliative	Death <6/12
M	85	211	T_3G_3	Palliative	Death <2/12
F	84	170	T_2G_3	None	Clear 12/12
M	71	164	T_2G_3	Radiotherapy	Metastases 15/12
M	82	136	T_2G_3	Radiotherapy	Recurrence 9/12
F	74	104	T_4G_3	Radiotherapy	Death <10/12
M	30	92	T_4G_3	Cystectomy + chemotherapy	Death <12/12
F	75	92	T_3G_3	Radiotherapy	Death <12/12
M	68	79	T_2G_2	Radiotherapy	Recurrence 1 y
M	75	71	T_4G_3	Radiotherapy	Death <3/12
M	69	57	T_3G_2	Radiotherapy	Metastases 12/12
F	77	52	T_2G_2	Radiotherapy	Death 18/12
M	78	50	T_3G_2	Radiotherapy	Clear 12/12
M	74	40	T_4G_3	None	Death 3/12
M	75	29	T_3G_2	Radiotherapy	Clear 18/12
M	73	27	T_3G_3	Cystectomy	Clear 15/12
M	72	25	T_2G_3	None	Clear 18/12
F	79	24	T_3G_3	Radiotherapy	Death 6/12
F	73	8	T_3G_3	Radiotherapy	Clear 12/12
M	59	7	T_4G_3	Palliative	Death <2/12
M	72	7	T_3G_3	Radiotherapy	Clear 24/12

[a] M, male; F, female.

here suggest that MK may be operational as an angiogenic factor in both pathways. It seems reasonable that the simultaneous production of two or three angiogenic factors in a tumor will lead to a more highly angiogenic, and possibly more aggressive, phenotype than that induced by a single factor. Simultaneous production of different angiogenic factors in a single tumor makes the therapeutic goal of preventing angiogenesis by the inhibition of a single angiogenic factor seem more unlikely (18), although different angiogenic factors may control key stages of the angiogenic process.

The cell type responsible for the MK expression in these bladder tumors is not known but may well be the tumor cells themselves, because we have found strong MK mRNA expression in both the 253J and T24 bladder cell lines, which are both derived from invasive, highly aggressive tumors (data not shown). By contrast, the RT4 line, which serves as a paradigm of well-differentiated tumors, does not express MK. Formal localization studies using immunohistochemistry or *in situ* hybridization need to be performed.

A finding of particular interest in this study is that patients with invasive bladder tumors that express little or no MK tend to have a better outcome at 1 year than patients whose tumors overexpress MK. In five (70%) of the seven patients who are alive with no evidence of disease, MK expression in the tumors was within the normal range at the time of presentation. The reason for the improved outcome is not clear but would not appear to be the result of any sampling bias in favor of patients with T_2 tumors. One explanation might be that the absence of MK expression leads to poor vascularization of a tumor, low vascular density, and, therefore, a more favorable outcome. We have not measured vascular densities in this group of tumors, but it would be of interest to ascertain whether MK expression correlated with vascular density. An alternative explanation for the association between overexpression of MK and an unfavorable clinical outcome is suggest by *in vitro* work, in which MK has been shown to induce plasminogen activators in aortic endothelial cells (19); activation of plasminogen activators within a tumor is known to facilitate tumor progression and metastasis, and overexpression of urokinase type plasminogen activators by superficial bladder tumors is associated with a increased chance of local recurrence (20).

In contrast to the situation with invasive cancers, MK expression appeared not to be a key determinant of the potential for recurrence of superficial cancers. The explanation for this is not clear, but it may be that MK receptors are differentially expressed in superficial and invasive cancers. Alternatively, MK might synergize more effectively with the other factors responsible for angiogenesis in invasive tumors than it does with the factors implicated in angiogenesis in superficial tumors.

The study group in our series of invasive cancers is admittedly small (22 tumors), and a larger group of invasive tumors needs to be examined to clarify the relationship between prognosis and MK expression. Aside from conventional pathological staging and grading, the best current means of differentiating invasive cancers with a good prognosis from those with a poor prognosis are by estimations of p53 status, epidermal growth factor receptor status, and vascular density. It remains to be seen whether estimates of MK expression prove to be useful adjuncts to these other molecular prognostic tools.

Noninvasive diagnostic methods in bladder cancer are still imperfect, largely because of the wide differences in levels of expression of potential markers between different tumors. Because MK is expressed by 97% of bladder cancers, it might be of interest in the future to attempt to detect MK in urine and to assess its potential as a noninvasive marker of disease activity. Acidic and basic FGFs (15, 16) and VEGF[5] are all detectable in urine, but there are no reports to date concerning urinary MK. Antibodies to MK are available and could be used for Western analysis of concentrated urine.

This study has demonstrated overexpression of MK in bladder cancers and has demonstrated a link between poor outcome in patients with invasive cancers and the level of MK expression. MK is worthy of further study in bladder cancer.

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[5] T. O'Brien, unpublished observations.