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Overexpression of midkine in lung tumors induced by *N*-nitrosobis(2-hydroxypropyl)amine in rats and its increase with progression

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The expression of midkine (MK) in lung tumors induced by *N*-nitrosobis(2-hydroxypropyl)amine (BHP) in rats was examined. The animals were administered 2000 p.p.m. of BHP in their drinking water for 12 weeks, then maintained without further treatment until being killed 20–28 weeks after the beginning of the experiment. MK mRNA expression of adenocarcinomas and squamous cell carcinomas assessed by means of the reverse transcriptase–polymerase chain reaction and northern blot analysis was significantly higher than in rat embryonic tissues (positive controls) and contrasted strongly with the lack in normal lungs. MK protein was detected immunohistochemically in 58.3% of alveolar hyperplasias, 92.3% of adenomas and 100% of adenocarcinomas and squamous cell carcinomas. The extent of staining significantly increased along with malignant progression in adenomatous (pre-)neoplastic lesions and tended to become more pronounced with malignant progression in squamous lesions. The results suggest that MK may play some essential roles in the development and progression of lung tumors induced by BHP in rats.

Introduction

Growth factors, involved in angiogenesis, have been shown to play important roles in the development of neoplasms (1–5). They may be promoted by either direct mitogen effects on cancer cells or by an indirect influence on stromal and vascular elements (6). Changes may occur in co-ordinating interactions between tumor parenchymal and non-parenchymal tissues, and growth factors play regulatory roles in such events (7–11). Fibroblast growth factors, hepatocyte growth factor and platelet-derived growth factors share one particular characteristic in that their biological functions are modulated by their binding to and release from heparan sulfate, a heparin-related carbohydrate molecule existing on the cell surface and in extracellular matrices, which ensures their location in sites of action for appropriate periods, and they are thus called heparin-binding growth factors (12).

Abbreviations: BHP, *N*-nitrosobis(2-hydroxypropyl)amine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MK, midkine; RT–PCR, reverse transcriptase–polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

Midkine (MK) was originally identified as the product of a retinoic acid-responsive gene (13,14) and was then recognized to be a novel heparin-binding growth factor. Since it is strongly expressed in the mid-gestation period of mouse embryogenesis (15–17), MK was initially considered to solely regulate events in the differentiation and development of organs (18–20). More recently, it was shown that it also played important roles in tissue remodeling and angiogenesis through enhancing plasminogen activators (21). MK is normally highly expressed in small bowel epithelium, moderately expressed in the thyroid and is present only at a low level in lung, stomach, colon and kidney in man (26,29). Expression has frequently been reported in various human tumors, such as cancers of the gastroenteral tract from the esophagus to rectum, the liver, pancreas, kidney, urinary bladder, lung and mammary gland, as well as neuroblastomas and astrocytomas in the brain (22–29). Especially in neuroblastomas and bladder carcinomas, the strength of MK expression correlates negatively with the patient's prognosis (23,28). Furthermore, an altered splicing form of MK mRNA has been detected in various human cancers (27,30–32). However, details of the roles of MK in carcinogenesis are still largely obscure and studies in animal models have so far been limited (33–38).

We have established a model for development of non-small cell lung carcinomas in rats by giving *N*-nitrosobis(2-hydroxypropyl)amine (BHP) in the drinking water, in which high yields of adenomatous and squamous pre-neoplastic and malignant lesions are induced (39,40). This model allows investigation of the molecular mechanisms that underlies the step-wise cancer development from the stage of early pre-neoplasia to advanced carcinomas, and is thus suitable to assess the contribution of MK to neoplasia in the lung. In the present study, we investigated MK expression in this model using reverse transcriptase–polymerase chain reaction (RT–PCR), northern blot analysis and immunohistochemical staining.

Materials and methods

Animals and treatments

A total of 35 male Wistar rats and one pregnant female Wistar rat were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan) at an age of 5 weeks, and were maintained under constant conditions of 25 ± 3°C, 55 ± 8% humidity and a 12 h dark–light cycle with free access to a basal diet (CE-2 diet; Clea Japan, Meguro, Tokyo, Japan) and drinking water throughout acclimation (1 week) and the experimental periods. After acclimatization, the males were divided into two groups. Group 1 consisted of 25 rats who received 2000 p.p.m. BHP (Nacalai Tesque, Kyoto, Japan) in drinking water for 15 weeks, and then received drinking water without BHP for 5–13 weeks. Group 2 consisted of 10 rats who were given drinking water without BHP throughout the experiment. The animals were killed under light ether anesthesia 20–28 weeks after the beginning of the experiment and the lungs were removed, fixed in 10% buffered formalin and routinely processed for embedding in paraffin and MK immunohistochemistry as described below. Additionally, tumors >5 mm in diameter were immediately excised and divided into two parts. One half was frozen in liquid nitrogen and stored at –80°C until use for RNA analyses. The remaining halves were made into paraffin blocks as above for histological evaluation using routine hematoxylin and eosin staining

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and for MK immunohistochemistry. Diagnostic criteria for lung lesions were as previously described (39,40). For positive controls in the RNA analyses, five embryos were taken from the one pregnant rat at 14 days of gestation under light ether anesthesia and her whole tissue was immediately frozen in liquid nitrogen and stored at –80°C until use.

RNA isolation and RT–PCR analysis

Using an ISOGEN kit (Nippon Gene, Toyama, Japan), total cellular RNA was extracted from five examples, each of whole embryonal tissues, normal lung tissues, adenocarcinomas and squamous cell carcinomas, according to the manufacturer's instructions. cDNA from total RNA was synthesized with a Superscript pre-amplification system (Gibco BRL, Life Technologies, Rockville, MD); briefly, a 3 mg aliquot of total RNA with 0.5 mg of oligo(dT) in diethylpyrocarbonate-treated water was incubated at 70°C for 10 min and on ice for 2 min. Then 7 µl of reaction mixture (2 µl 10× PCR buffer, 2 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTP mix, 2 µl 0.1 M dithiothreitol) was added to each sample and mixed, followed by incubation at 42°C for 5 min. An aliquot of 1 µl (200 U) of SuperScript II RT was added and mixed, incubation was continued at 42°C for 50 min, and the reaction was terminated by heating to 70°C for 15 min. Then, first strand cDNA was purified by digestion with 1 µl RNase H, and was used as a PCR template. Aliquots of 1 µl of 10-fold diluted cDNA solutions were subjected to PCR in 20 µl (2 µl 10× PCR buffer; 2 µl dNTP mix; 0.1 µl Taq DNA polymerase; 0.2 µl primers; 14.7 µl autoclaved, distilled water). Appropriate oligonucleotides for specific amplification of MK cDNA (13) were prepared with sequences of 5′-ATGCAGCACCGAGGCTTCTT-3′ as the forward primer and 5′-GTC-CTTTCCTTTTCCTTTCT-3′ as the reverse primer. PCR was conducted with a GeneAmp DNA Amplification Reagent kit (Perkin-Elmer Cetus, Norwalk, WI) and Taq polymerase (Pharmacia Biotech, Uppsala, Sweden) with the following conditions; pre-heating at 95°C for 1 min, 35 cycles of 30 s denaturation at 94°C, a 60 s annealing at 58°C, a 60 s extension at 72°C and an 8 min post-extension at 72°C. The resultant PCR products were then electrophoresed in 1.2% agarose gels.

Northern blot analysis

Northern blot analysis was performed for four embryonal tissues, five adenocarcinomas, five squamous cell carcinomas and five normal lung tissues in which MK mRNA was detected by RT–PCR. Aliquots of 20 µg RNA were electrophoresed in 1% agarose–formaldehyde gels and transferred to Biodyne A nylon membranes (Pall Biosupport, East Hills, NY). A 730 bp fragment of mouse MK cDNA (13) was generously provided by Dr K.Kadomatsu (Department of Biochemistry, Nagoya University School of Medicine, Aichi, Japan) and used as a probe. The probe was ^{32}P-radiolabeled using a DNA Labeling kit (d-CTP) (Pharmacia Biotech) in a mixture of 50% formamide, 5× standard saline citrate (SSC), 0.1 M phosphate buffer (pH 7.4), 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA and 100 mg/ml salmon testis DNA (Sigma, St Louis, MO) overnight at 45°C. The membranes were baked at 80°C for 1 h and hybridized with 1×10^6 c.p.m./ml of the labeled probe, and blots were washed once in 2× SSC and 0.1% SDS at room temperature for 15 min and twice in 2× SSC and 0.1% SDS at 55°C for 15 min followed by exposure of Hyper-film-MP (Amersham Japan, Tokyo, Japan) with an intensifying screen overnight at 80°C. After the exposure, the northern blots were reprobed with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe to confirm that similar amounts of RNA had been loaded and transferred from each sample. Densitometric analysis of gene expression was then performed with a BAS 1000 image analyzer (Fuji Photo Film, Tokyo, Japan) as previously described (43,44).

Immunohistochemical analysis

The immunohistochemical demonstration of anti-MK protein binding was achieved with a rabbit polyclonal anti-MK antibody (a gift from Dr K.Kadomatu) and a LSAB 2 kit (Dako Japan, Kyoto, Japan) with visualization of the binding using 3,3′-diaminobenzidine tetrahydrochloride. Staining intensities were classified according to the proportions of positive cells, as: negative, none; slightly positive, <50%; positive, 50–90%; and strongly positive, >90%. The specificity of the binding was confirmed by negative control staining using rabbit non-immune serum instead of the primary antibody (data not shown).

Statistics

Statistical analyses were routinely performed using a personal computer as described elsewhere (41) for the levels of MK mRNA expression in embryonic tissues (positive controls) and for tumors using the Mann–Whitney *U*-test (two-tailed). Incidences of immunohistochemically demonstrated MK protein expression were evaluated with the χ^2 test.

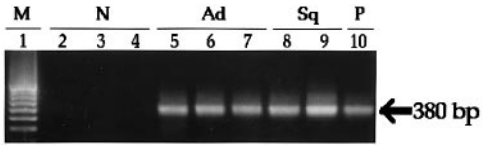

Fig. 1. Representative radiographical findings from RT–PCR analysis of MK mRNA in BHP-induced rat lung tumors. Arrows indicate the mature form of MK. Lane 1 (M), 100 bp ladder as the size marker; lanes 2–4 (N), normal lung tissues; lanes 5–7 (Ad), adenocarcinomas; truncated form is in evidence; lanes 8–9 (Sq), squamous cell carcinomas; lane 10 (P), embryonal tissue (positive control).

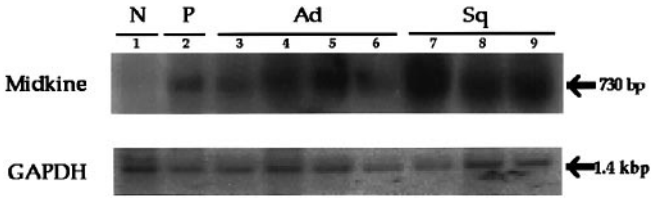

Fig. 2. Representative radiographical findings from northern blot analysis of MK gene expression in BHP-induced rat lung tumors. The GAPDH probe was co-hybridized to confirm the same amount of mRNA loading for each lane. Lane 1, normal lung tissue; lane 2, embryonic tissue (positive control); lanes 3–6, adenocarcinomas; lanes 7–9, squamous cell carcinomas.

Results

RT–PCR analysis for MK mRNA expression

The expression of MK mRNA was initially evaluated by RT–PCR with cDNA prepared from frozen sections of adenocarcinomas and squamous cell carcinomas, the representative radiographical findings are presented in Figure 1. Adenomas and squamous metaplasias could not be assessed because of limited tissue availability. Oligonucleotide primers in this study were used to amplify a 380 base cDNA for mouse MK2 (13). All of the analyzed adenocarcinomas and squamous cell carcinomas, as well as the embryonal tissues used as positive controls, produced products that corresponded to the expected PCR bands. Truncated variant forms of MK were not detected in any specimens. No PCR band was detected in the normal tissue case (Figure 1).

Northern blot analysis for MK mRNA expression

The MK mRNA detected by RT–PCR in adenocarcinomas and squamous cell carcinomas was quantified by northern blot hybridization of total RNA extracted from such tumors; representative radiographical findings are shown in Figure 2. The 730 bp MK mRNA was strongly expressed in all of the adenocarcinoma and squamous cell carcinoma samples but not in any of normal lung tissues examined. The results of the MK gene quantification by densitometry with a normalization using the GAPDH value are summarized in Table I. MK transcript levels of adenocarcinomas and squamous cell carcinomas were both significantly higher than those of embryonal tissues.

Immunohistochemical staining for MK protein

Positive anti-MK protein binding was immunohistochemically demonstrable in the cytoplasm of tumor cells (Figure 3) but not normal lung tissue (data not shown). Many of the alveolar hyperplasias showed negative (41.7%) or slightly positive (38.5%) staining and no lesions were strongly positive (Table II). In contrast, 34.6% of adenomas were strongly positive stained (Figure 3A) and only 7.7% were negative. The staining extent of adenomas and adenocarcinomas was significantly greater than that of alveolar hyperplasias

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Table I. Densitometrically quantified data for MK mRNA expression in BHP-induced rat lung tumors

Histology	Sample No.	Midkine mRNA expression[a] (arbitrary units)
Embryonal tissue	1	1.15
	2	1.17
	3	1.08
	4	1.18
	mean ± standard deviation	1.41 ± 0.05
Adenocarcinoma	5	5.00
	6	6.97
	7	8.28
	8	5.65
	9	6.25
	mean ± standard deviation	6.43 ± 1.26[b]
Squamous cell carcinoma	10	8.81
	11	9.33
	12	8.87
	13	8.10
	14	7.87
	mean ± standard deviation	8.60 ± 0.60[b]

[a]Normalized to GAPDH values.
[b]Significantly higher than the embryonal tissue level ($P<0.05$).

Table II. Summary of immunohistochemical findings for MK protein expression in BHP-induced rat lung tumors

Histology	Total number of lesions examined	Number of lesions belonging to each category of staining extent[a]			
		Negative	Slightly positive	Positive	Strongly positive
Alveolar hyperplasia	96	40 (41.7)	37 (38.5)	19 (19.8)	0 (0)
Adenoma	26	2 (7.7)	8 (30.8)	7 (26.4)	9 (34.6)
Adenocarcinoma	27	0 (0)	1 (3.7)	3 (11.1)	23 (85.2)
Squamous metaplasia	10	0 (0)	1 (10.0)	9 (90.0)	0 (0)
Squamous cell carcinoma	8	0 (0)	1 (12.5)	0 (0)	7 (87.5)

[a]Numbers in parentheses represent percentage incidences.

($P < 0.001$). Furthermore, the value for adenocarcinomas was significantly greater than that for adenomas ($P < 0.001$). Although all squamous metaplasias were stained, none exhibited strong positivity (Table II). In contrast, 87.5% of squamous cell carcinomas were strongly positive (Figure 3C, Table II). It should be noted that all the cancers, for which MK mRNA expression was detected, demonstrated strongly positive anti-MK protein binding.

Discussion

The present study clearly indicates the possible expression of MK mRNA and protein in BHP-induced lung adenocarcinomas and squamous cell carcinomas in rats, in contrast to the lack in normal tissue. MK protein expression increased with progression from alveolar hyperplasia to adenoma, and from adenoma to adenocarcinoma. A similar, but less pronounced, increase was also apparent with the transition from squamous cell metaplasia to squamous cell carcinomas. In both cases, levels were higher than in embryonal tissues assayed as positive controls. MK has been reported to serve as an autocrine mitogen for G401 cell line from a rhabdoid kidney tumor (22). This was thought to be mediated by a specific cell-surface receptor, which, in turn, activates a pathway consisting of Janus tyrosine kinases and signal transducers and activators of transcription (36). The architecture of the MK receptor complex, as well as the molecular mechanisms underlying the MK-induced signal transduction, however, remain to be elucidated. Angiogenesis is a prerequisite for tumor growth (42) and Choudhuri *et al*. have described that overexpression of MK lead to endothelial growth-stimulating activity in MCF-7 breast carcinoma cells transfected with MK mouse cDNA that was cloned into plasmid DNA Ineo, which enhanced both tumor growth and vascular density (34). Taken together, the data suggested that MK may contribute to tumor growth and progression by virtue of its direct mitogenic and angiogenic effects. It is well known that various factors participate in tumor angiogenesis (1–5), but little is known about their relative importance or interactions with regard to angiogenic mechanisms. In human lung cancers, expression of vascular endothelial growth factor and pleiotrophin have been demonstrated (25,43). The present results suggest that MK may be expressed as an angiogenic factor in BHP-induced rat lung tumorigenesis, and thus work with other factors to enhance malignant potential.

Altered forms of growth factors or tumor suppressor gene products produced in tumor cells by alternative splicing are suggested to play important roles in tumorigenesis (30–32,45). Similarly, in the case of MK, it has recently been demonstrated that a truncated type of MK transcript, which encodes an alternatively spliced product that lacks the third exon, is expressed in pancreatic carcinoma cell lines (30), human colorectal (31) and breast cancers (32), but not in normal tissues (30–32). Because of the lack of an N-terminally located domain, the truncated form of MK is thought to lack a region

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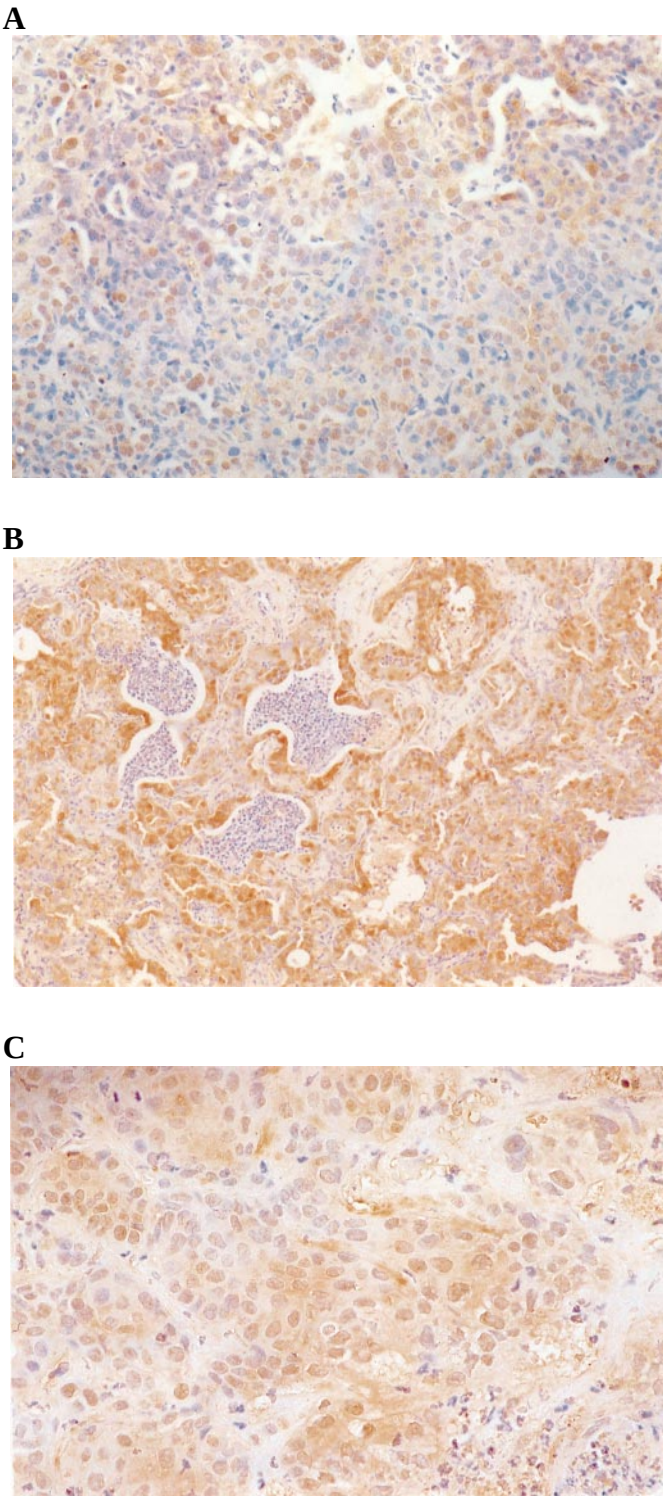

Fig. 3. Representative immunohistochemical demonstration of anti-MK binding in BHP-induced rat lung tumors. (**A**) Adenoma, (**B**) adenocarcinoma, (**C**) squamous cell carcinoma.

that is required for its proper localization in extracellular matrices, which results in disorganized cellular activity (30). Our RT–PCR analysis, however, did not reveal any truncated-type MK transcripts in the adenocarcinomas and squamous cell carcinomas examined. Therefore, alternative processing of MK mRNA may not contribute to BHP-induced development of lung tumors in rats.

Whereas progression of *N*-nitroso-*N*-methylurea-induced rat mammary tumors has been associated with a loss of the *MK* gene (33), expression has been observed from the early through to the terminal stages of diethylnitrosamine-induced rat liver carcinogenesis (Kanda *et al*., manuscript in preparation). In the present case of BHP-induced rat lung carcinogenesis, MK was found to be expressed in more than half of the alveolar hyperplasias, most adenomas and all adenocarcinomas, as well as all squamous metaplasias and squamous cell carcinomas. Moreover, in both cases, the extent of immunohistochemical staining increased along with the malignant nature of the lesions. We are now focusing attention on the detailed mechanisms that underly MK expression in carcinogenesis.

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