

## Overexpression of Midkine in Pancreatic Duct Adenocarcinomas Induced by *N*-Nitrosobis(2-oxopropyl)amine in Hamsters and Their Cell Lines

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The expression of midkine (MK) was investigated in pancreatic ductal hyperplasias, atypical hyperplasias and adenocarcinomas induced by *N*-nitrosobis(2-oxopropyl)amine (BOP) in hamsters, and in hamster ductal adenocarcinoma cell lines (HPD-1NR, -2NR and -3NR). MK mRNA was clearly overexpressed in invasive pancreatic duct adenocarcinomas (PCs) and the three cell lines as assessed by northern blot analysis, and MK protein expression increased from ductal hyperplasia through atypical hyperplasias, intraductal carcinomas and invasive PCs by immunohistochemistry. The extent of overexpression of MK mRNA in PCs was almost the same as in hamster whole embryonic tissue. MK is reported to be a retinoid-responsive gene, but MK mRNA expression was not affected by treatment with all-*trans* retinoic acid (tRA) or *N*-(4-hydroxyphenyl)retinamide (4-HPR) in HPD-1NR cells. The results thus suggest that MK expression is involved in the development and progression of pancreatic ductal adenocarcinomas induced by BOP in hamsters, with loss of upregulation by retinoic acid.

Key words: Midkine — Pancreatic duct carcinogenesis — Hamster — 4-HPR — tRA

Pancreatic duct carcinoma (PC) is the fifth leading cause of cancer death in both the US and Japan.<sup>1,2</sup> Due to its silent clinical course, at the time of diagnosis the vast majority of pancreatic cancer cases are incurable, with a very poor prognosis. In order to control the disease, it is indispensable to detect tumors as early as possible and then to undertake measures to prevent subsequent progression. An experimental model suitable for investigation of human PC development has been established in hamsters using the carcinogen *N*-nitrosobis(2-oxopropyl)amine (BOP) and related compounds.<sup>3–5</sup> To facilitate studies of the underlying mechanisms, we have developed a rapid production model of PCs,<sup>6–8</sup> incorporating the principle of selection by resistance to cytotoxicity demonstrated earlier for liver carcinogenesis in rats.<sup>9,10</sup> Further, we have established PC cell lines and transplantable PC from the resultant cancers.<sup>11,12</sup> This model provides a whole sequence of lesions involved in pancreatic duct carcinogenesis, not only facilitating understanding of factors contributing to tumor induction, but also serving as a bioassay for identification of appropriate chemopreventive or chemotherapeutic agents.<sup>13</sup>

Midkine (MK) was originally identified as the product of a retinoic acid-responsive gene<sup>14,15</sup> and then recognized

to be a novel heparin-binding growth factor. Since it is strongly expressed in the midgestation period of mouse embryogenesis,<sup>16–18</sup> MK was initially considered to regulate solely events in the differentiation and development of organs.<sup>14,19,20</sup> More recently, however, it was also shown to play important roles in tissue remodeling and angiogenesis through enhancing plasminogen activators.<sup>21</sup> While MK is normally strongly expressed in the small bowel epithelium and moderately in the thyroid, it is present at only low levels in the lung, stomach, colon, and kidney in man.<sup>22,23</sup>

Elevated expression has frequently been reported in various human tumors such as cancers of the gastrointestinal 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.<sup>23–28</sup> Especially in neuroblastomas and bladder carcinomas, the strength of MK expression correlates negatively with the patient's prognosis.<sup>24,28</sup> Recently, we reported overexpression of MK in lung tumors induced by *N*-nitrosobis(2-hydroxypropyl)amine in rats and its increase with progression. However, to clarify the role of MK during carcinogenesis, data are needed from animal models.<sup>29,30</sup>

In the present study, we therefore investigated MK expression in the pancreatic carcinogenesis model using northern blot analysis and immunohistochemical staining.

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**MATERIALS AND METHODS**

**Animals and treatments** Female Syrian golden hamsters (Nihon SLC, Shizuoka) weighing approximately 100 g each (7 weeks old) were used. Pancreatic duct carcinomas were induced by the rapid-production model,<sup>6)</sup> with the protocol shown in Fig. 1. This consists of an initial subcutaneous injection of 70 mg/kg body weight BOP (Nacalai Tesque, Kyoto) followed 12 days later by three cycles of ethionine-methionine rescue-induced pancreatic regeneration. During this time, the hamsters were maintained on a choline-methionine-deficient diet. Each switch to methionine was followed after 2 days by an injection of 20 mg/kg BOP, and the cycles were separated from one another by an interval of 10 days. The hamsters were killed under ether anesthesia 12–15 weeks after the beginning of the experiment and each pancreas was removed, fixed in 10% buffered formalin and routinely processed for embedding in paraffin for histological examination. When tumors were detected macroscopically, a portion of the tumor (weighing more than 50 mg) was resected and immediately frozen in liquid nitrogen for RNA extraction. The remaining halves were made into paraffin blocks for histological evaluation using routine hematoxylin and eosin staining and for MK immunohistochemistry. Diagnostic criteria for ductal lesions were as previously described.<sup>6, 12)</sup> For the analysis of the normal distribution of MK in the hamster, three embryos were taken from one pregnant hamster (Nihon SLC, Shizuoka) at 14 days of gestation under ether anesthesia and whole embryo tissues were frozen immediately in liquid nitrogen. Three newborn animals, taken from one litter, and three young-adult (7

weeks old) hamsters, which were purchased from Nihon SLC, were killed under ether anesthesia and the pancreas, kidney, liver, lung and brain were removed and frozen immediately in liquid nitrogen.

**RNA isolation and northern analysis** Total RNA was extracted from hamster embryo, normal kidney, liver and PC tissue by the lithium chloride-urea method,<sup>31)</sup> and from normal pancreas tissue by the phenol-proteinase K method,<sup>32)</sup> as previously described. Using an ISOGEN kit (Nippon Gene Co., Ltd., Toyama), total cellular RNA was extracted from HPD cell lines according to the instructions of the manufacturer. Northern blot analysis was performed for 10 PCs, 3 embryos at day 14 of gestation and normal pancreas, liver and kidney tissues of 3 newborns. For northern analysis, 25 µg of total RNA extracted from tissue samples and 10 µg of total RNA extracted from HPD cells were used. Aliquots of total RNA were electrophoresed in 1% agarose-formaldehyde gels and transferred to Hybond-N nylon membranes (Amersham Intl. Plc, Amersham, UK). A 730-bp fragment of mouse MK cDNA was used as a probe<sup>13)</sup> <sup>32</sup>P-radiolabeled using a DNA Labeling Kit (d-CTP) (Pharmacia Biotech, Uppsala, Sweden) and hybridized 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 ethylenediaminetetraacetic acid (EDTA), and 100 mg/ml salmon testis DNA (Sigma Chemical Co., St. Louis, MO) at 37°C for 36 h. Then membranes 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. The hybridized membranes were exposed to Fuji Bio-Imaging plates (Fuji Photo Film Co., Ltd., Tokyo) and quantification of gene expression was performed with a Fujix MacBas1000 phosphoimager (Fuji Photo Film Co., Ltd.). To normalize the amounts of RNA blotted, the northern blots were rehybridized with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe.

**Immunohistochemical analysis** The method of immunohistochemical demonstration of anti-MK protein binding, using a rabbit polyclonal anti-MK antibody and a LSAB 2 kit (DAKO Japan, Kyoto) with visualization of the binding using 3,3'-diaminobenzidine tetrahydrochloride was described previously.<sup>29)</sup> Staining intensity was classified according to the population of positive cells as: negative, no positive cells; slightly positive, less than 30% ; positive, more than 30%. The specificity of the binding was confirmed with negative control staining using rabbit non-immune serum instead of the primary antibody.

**Cell lines and treatment of all-trans retinoic acid (tRA) or N-(4-hydroxyphenyl)retinamide (4-HPR)** Three hamster PC cell lines (HPD-1NR, 2NR and 3NR) established in our laboratory<sup>11)</sup> were used in this study. These cell lines were derived from a transplantable PC that origi-

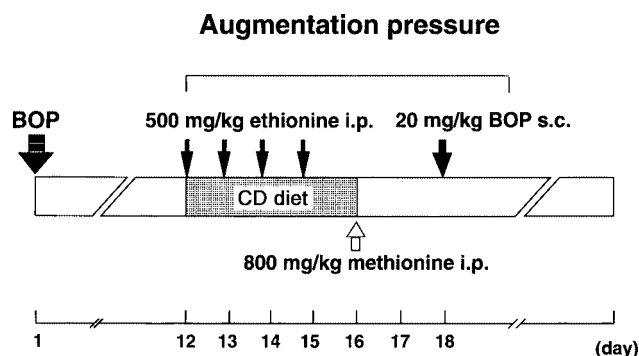


Fig. 1. Basic protocol for rapid production of PCs in hamsters. As initiation, 70 mg/kg BOP was injected subcutaneously followed by augmentation pressure. The following treatment is involved in augmentation pressure as shown in Fig.1; basal diet was switched to choline-deficient (CD) diet and 500 mg/kg ethionine was injected daily 4 times, then CD diet was switched to basal diet and 800 mg/kg methionine was injected. Two days after methionine injection, 20 mg/kg BOP was injected.

nated from an invasive PC induced by *N*-nitrosobis(2-hydroxypropyl)nitrosamine (BHP). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui Seiyaku, Tokyo) supplemented with 10% fetal bovine serum (FBS, Flow Lab., McLean, VA). tRA was obtained from Nacalai Tesque and 4-HPR was generously supplied by the R. W. Johnson Pharmaceutical Research Institute (Spring House, PA). To study upregulation of MK expression by retinoic acid, HPD-1NR was cultured in medium containing 0, vehicle alone, 5, 10  $\mu$ M tRA or 2, 5  $\mu$ M 4-HPR and harvested at 1 and 2 days. tRA and 4-HPR were dissolved in ethanol as 10 mM stock solutions and added to DMEM containing 10% FBS before use. After trypsinization, cells were collected and frozen in liquid nitrogen for RNA extraction. To test for proliferation, cells were cultured in medium containing tRA or 4-HPR and MTS assays were performed at 1, 2, 3 and 5 days according to the manufacturer's instructions for Cell Titer 96 (Promega, Madison, WI). Briefly, MTS solution was added to each sample and incubation was conducted for 1 h at 37°C. Then, the value of absorption at 490 nm was measured with a UV spectrophotometer.

**Statistics** Incidences of immunohistochemically demonstrated MK protein expression were statistically evaluated with the  $\chi^2$  test.

## RESULTS

**Northern blot analysis of MK mRNA expression** Faint MK mRNA expression was detected in the normal pancreas and kidneys of newborn hamsters, and expression was strong in the embryos, all of 10 PCs and three HPD cell lines. Representative radiographic findings are shown in Fig. 2. In the pancreas, liver, kidney, lung and brain of young-adult hamsters, MK mRNA expression was not detectable. The results of the *MK* gene quantification by densitometry with normalization to the GAPDH value are summarized in Table I. Relative signal intensities were clearly high in the embryos, PCs and three cell lines.

**Immunohistochemical staining of MK protein** In normal pancreas of young-adult hamsters, islet cells were found to show weak positive staining for MK, whereas ductal and acinar cells were negative (Fig. 3a). The results of immunohistochemical staining are summarized in Table II. Almost all ductal hyperplasias were negative (82%) or only slightly positive (18%). In contrast, 25% of intraductal carcinomas and 75% of invasive ductal carcinomas were in the positive category and no PCs were negative. Anti-MK protein binding was also immunohistochemically demonstrated in the cytoplasm of all cancers (Fig. 3c). The expression level had a tendency to increase with malignant transformation and progression of PCs, and was significantly greater in invasive PCs than in hyperplasias and atypical hyperplasias ( $P < 0.001$ ). It should be noted here

that all of the PCs which showed increased MK mRNA expression were demonstrated to be positive for anti-MK protein binding.

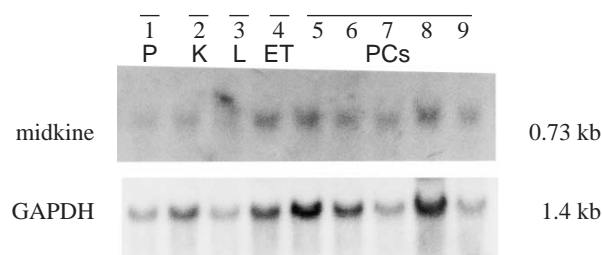


Fig. 2. Representative radiographic findings from northern blot analysis for MK expression in normal newborn pancreas (P, lane 1), kidney (K, lane 2), liver (L, lane 3), embryo (ET, lane 4), and pancreatic duct adenocarcinomas (PCs, lanes 5–9) of hamsters. The glyceraldehyde-3'-phosphate dehydrogenase (GAPDH) cDNA was re-hybridized to confirm equal RNA loading of the lanes.

Table I. Densitometric Data for MK mRNA Expression in Hamster Embryonic Tissue, Various Tissues of Newborns and Young Adults and Pancreatic Duct Adenocarcinomas and Cell Lines<sup>a)</sup>

Sample	No. examined	Relative signal intensity of MK expression <sup>b)</sup>
Embryo	3	0.41±0.01
Pancreas		
Newborn	3	0.07±0.01
Young-adult	3	ND <sup>c)</sup>
Kidney		
Newborn	3	0.09±0.01
Young-adult	3	ND
Liver		
Newborn	3	0.04±0.01
Young-adult	3	ND
Lung		
Newborn	3	ND
Young-adult	3	ND
Brain		
Young-adult	3	ND
Pancreatic duct adenocarcinoma	10	0.46±0.13
HPD cell lines	3	0.59±0.06

a) Normalized to the glyceraldehyde-3'-phosphate dehydrogenase (GAPDH) values.

b) Mean±SD.

c) ND: not detected.

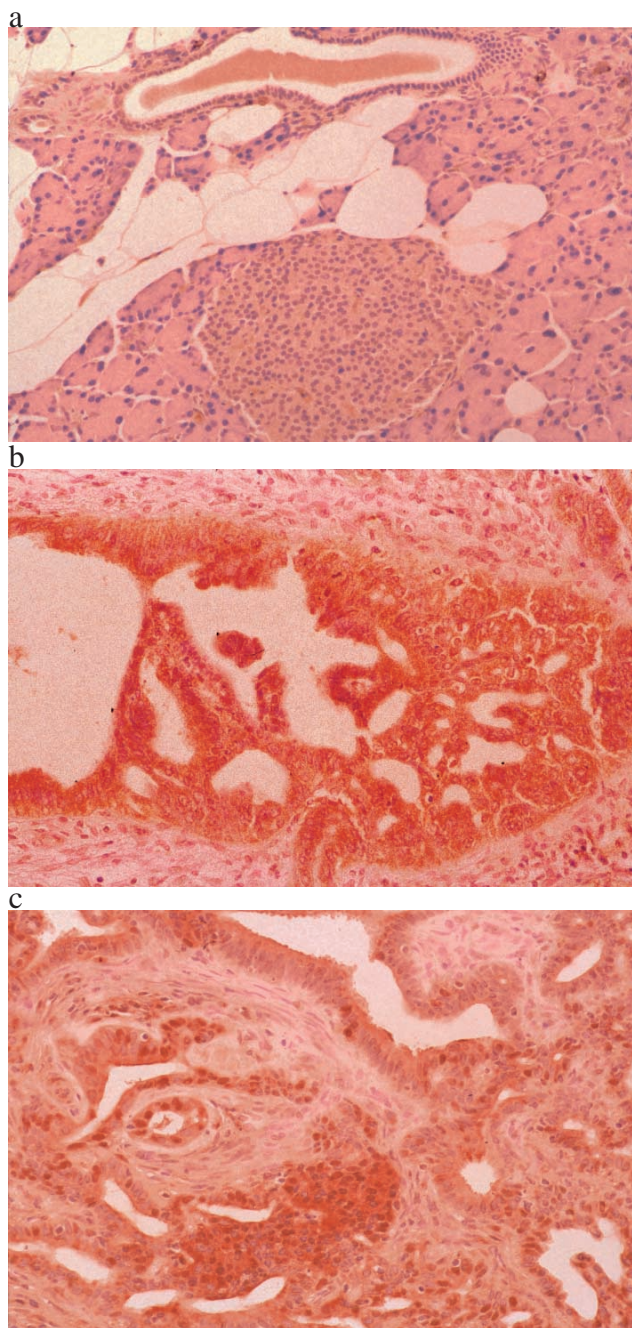


Fig. 3. Representative illustrations of immunohistochemical staining of MK in Syrian hamsters. (a) Normal pancreas of a young-adult hamster, (b) intraductal carcinoma of the pancreas, (c) invasive ductal adenocarcinoma of the pancreas.

**tRA and 4HPR inhibited cell proliferation, but not MK mRNA expression in cultured PC cells** The results of MTS assays and northern analysis of HPD-1NR cells

Table II. Summary of Immunohistochemical Findings for MK Protein Expression in Pancreatic Ductal Lesions Induced by BOP and Augmentation Pressure in Hamsters

Ductal lesions	Total number of lesions examined	No. of lesions belonging to each category of staining extent <sup>a)</sup>		
		Negative	Slight positive	Positive
Hyperplasia	45	40 (82)	8 (18)	0 (0)
Atypical hyperplasia	21	9 (43)	12 (57)	0 (0)
Intraductal carcinoma	4	0 (0)	3 (75)	1 (25)
Invasive ductal carcinoma	20	0 (0)	5 (25)	15 (75) <sup>b)</sup>

a) No. in parentheses are percentage incidences.

b)  $P < 0.001$  as compared with hyperplasia or atypical hyperplasia.

treated with various doses of tRA or 4-HPR for 5 days are shown in Figs. 4 and 5. With tRA, doses of 10 and 50  $\mu\text{M}$  inhibited proliferation by approximately 50% and 80%, respectively, but without any influence on the expression level of MK mRNA. 4-HPR at 5  $\mu\text{M}$  inhibited cell proliferation, but not MK expression, and at 10 and 50  $\mu\text{M}$  induced cell death.

## DISCUSSION

It has been reported that growth factors and their receptors play important roles in cell proliferation and acquisition of malignant potential of pancreatic carcinomas.<sup>33-37</sup> Epithelial growth factor (EGF), transforming growth factor (TGF)- $\alpha$ , insulin-like growth factor and their receptors,<sup>33-35</sup> and hepatocyte growth factor (HGF) and c-met<sup>36, 37</sup> are expressed in human cultured pancreatic cancer cells and tissues, suggesting that they contribute in an autocrine fashion to growth of pancreatic cancer cells. In the present study, MK mRNA and protein were expressed in hamster PC tissues and cell lines. The MK receptor has not been unequivocally identified, but MK binds to a high-affinity signaling receptor associated with JAK tyrosine kinase and serves as an autocrine mitogen for rhabdoid tumor cell line.<sup>38</sup> It was also reported that MK binds a receptor-like protein-tyrosine phosphatase  $\zeta$ <sup>39</sup> and activates mitogen-activated protein kinase and phosphatidylinositol 3-kinase in neuron cells.<sup>40</sup> To clarify the role of MK in the growth of PCs, further study is now needed of the expression of the MK receptor genes during pancreatic carcinogenesis.

MK protein expression increased with progression from ductal hyperplasias to invasive PCs and its expression was higher in invasive PCs than in intraductal, non-invasive PCs. Although the causal sequence of the MK overexpression in neoplasias remains to be elucidated, the present results suggest that MK might contribute to acquisition of



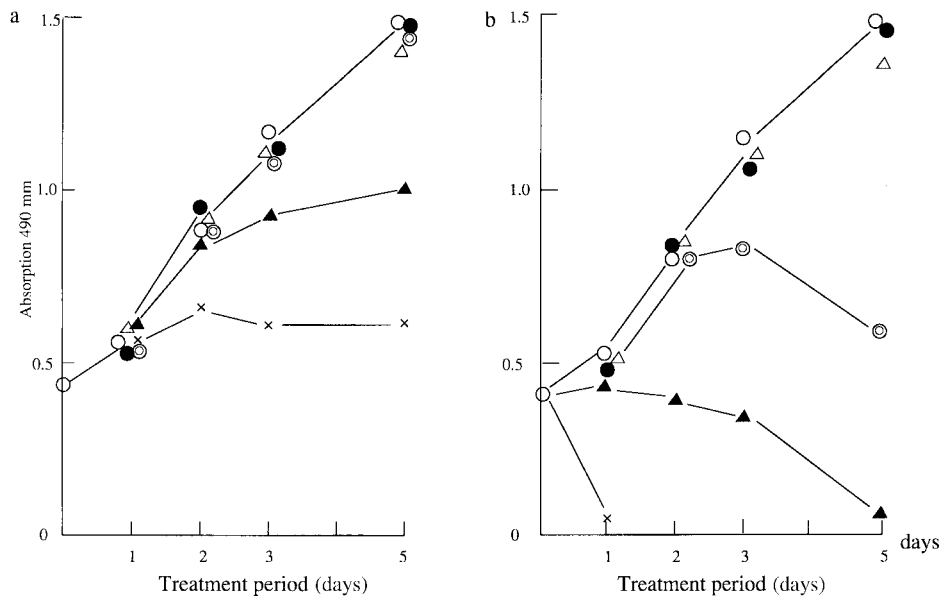


Fig. 4. Cell proliferation of HPD cells treated with tRA or 4HPR assessed by MTS assay. (a) Cells treated with tRA. Medium only, ○; vehicle, ●; 2 μM, △; 5 μM, ⊙; 10 μM, ▲; 50 μM, ×. (b) Cells treated with 4HPR. Medium only, ○; vehicle, ●; 2 μM, △; 5 μM, ⊙; 10 μM, ▲; 50 μM, ×.

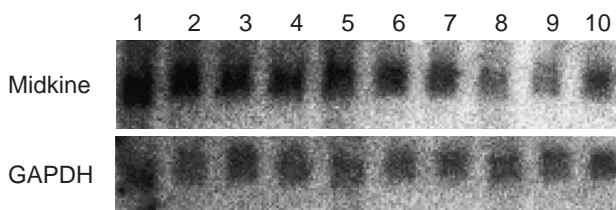


Fig. 5. Northern blot analysis of MK expression in HPD cells treated with tRA or 4HPR. Lane 1, 0 day; 2, cells treated with vehicle for 2 days; 3 and 4, cells treated with 5 and 10 μM tRA for a day; 5 and 6, cells treated with 5 and 10 μM tRA for 2 days; 7 and 8, cells treated with 2 and 5 μM 4HPR for a day; 9 and 10, cells treated with 2 and 5 μM 4HPR for 2 days.

malignant potential of preneoplastic pancreatic ductal cells. In fact, it was shown that NIH 3T3 cells transfected with an MK expression vector formed tumors in nude mice.<sup>39, 41</sup> MK also induces the migration of cells, such as neuronal cells<sup>39</sup> and smooth muscle cells.<sup>42</sup> In addition, MK has an anti-apoptotic activity through up-regulation of Bcl-2.<sup>43</sup> However, it has been reported that the *MK* gene is expressed in early but not late stages of mammary carcinogenesis by *N*-nitroso-*N*-methylurea in rats<sup>30</sup> and it is upregulated throughout liver carcinogenesis by diethylnitrosamine in rats (personal communication from Dr. T. Kanda, Cancer Institute, Tokyo). Thus, organ dependence

is indicated, but more data need to be accumulated in this context.

Retinoic acid is a molecule, which controls differentiation, growth and apoptosis in various kinds of malignant cells in animals and humans.<sup>44</sup> MK mRNA was earlier reported to be expressed after retinoic acid-induction of differentiation in a teratocarcinoma cell line within 1 day and in rat mammary carcinoma cells, where it was associated with reduced growth 2 days after the treatment with retinoic acid.<sup>30</sup> These findings suggest that MK mRNA expression is upregulated by retinoic acid. In fact, a retinoic acid-responsive enhancer was identified in the 5'-flanking region of the *MK* gene.<sup>45</sup> In the present experiment, we studied the effects of tRA, one physiologically active retinoid and 4-HPR, which is less toxic than tRA, and acts independently of the retinoid receptor.<sup>46</sup> The present finding of inhibition of HPD cell proliferation but unchanged MK mRNA expression by tRA and 4-HPR, thus, suggests a disturbance in the upregulation mechanisms in hamster PC cells. β-Carotene, which is a precursor of retinoic acid, exerts chemopreventive effects on pancreatic carcinogenesis in hamsters,<sup>47</sup> but this clearly might not involve regulation of MK expression.

It is now well recognized that angiogenesis plays important roles in development and progression of cancers and that various factors participate in this process.<sup>48</sup> Overexpression of MK leads to endothelial growth-stimulating activity in MCF-7 breast carcinoma cells transfected with

MK mouse cDNA, enhancing both tumor growth and vascular density.<sup>49)</sup> The data suggest that MK may contribute to tumor growth and progression by virtue of direct mitogenic and angiogenic effects. In hamster pancreatic cancer cells and human pancreatic ductal adenocarcinomas, expression of vascular endothelial growth factor (VEGF) has been demonstrated.<sup>13, 50)</sup> Thus, it has been reported that MK plays an important role in carcinogenesis by induction of malignant transformation, tumor cell proliferation, cell migration, anti-apoptotic activity and angiogenesis.<sup>39–43, 49)</sup> To clarify the role of MK in pancreatic carcinogenesis, transfection assay to artificially express MK in preneoplastic cells and to inhibit MK with anti-sense to MK in HPD cells should be performed.

Since MK is a secreted protein, the serum level of this molecule might serve as a tumor marker. In support of this idea, we recently reported that serum levels are significantly higher in cancer patients, including those with pan-

creatic carcinomas, than in healthy controls ( $P < 0.001$ ).<sup>51)</sup> Serum MK levels in fact decrease after surgical removal of tumors.<sup>51)</sup> The present findings suggest that overexpression of MK could serve as a potential marker for distinguishing benign and malignant lesions of pancreatic ducts.

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