

Review

Midkine, a heparin-binding cytokine with multiple roles in development, repair and diseases

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Abstract: Midkine is a heparin-binding cytokine or a growth factor with a molecular weight of 13 kDa. Midkine binds to oversulfated structures in heparan sulfate and chondroitin sulfate. The midkine receptor is a molecular complex containing proteoglycans. Midkine promotes migration, survival and other activities of target cells. Midkine has about 50% sequence identity with pleiotrophin. Mice deficient in both factors exhibit severe abnormalities including female infertility. In adults, midkine is expressed in damaged tissues and involved in the reparative process. It is also involved in inflammatory reactions by promoting the migration of leukocytes, induction of chemokines and suppression of regulatory T cells. Midkine is expressed in a variety of malignant tumors and promotes their growth and invasion. Midkine appears to be helpful for the treatment of injuries in the heart, brain, spinal cord and retina. Midkine inhibitors are expected to be effective in the treatment of malignancies, rheumatoid arthritis, multiple sclerosis, renal diseases, restenosis, hypertension and adhesion after surgery.

Keywords: midkine, pleiotrophin, proteoglycans, cancer, inflammation, repair

Introduction

Midkine (MK) is a cytokine or a growth factor and belongs to the carbohydrate-binding proteins.^{1)–3)} Cytokines and growth factors are classified to structurally-related protein families such as the fibroblast growth factor family. MK is the founding member of a family, which is composed of only two members in humans. The other member is pleiotrophin, also called HB-GAM.^{4),5)}

MK promotes growth,⁶⁾ survival,⁷⁾ migration⁸⁾ and gene expression⁹⁾ of various target cells. It is involved in reproduction¹⁰⁾ and repair,¹¹⁾ and also plays pathological roles in many diseases.

MK is attracting much attention in relation to the treatment of diseases. MK inhibitors are expected to be useful in treating cancer,¹²⁾ rheumatoid arthritis,¹³⁾ multiple sclerosis,¹⁴⁾ hypertension,¹⁵⁾ renal diseases⁹⁾ etc., while MK itself is promising for the treatment of ischemic brain injury,¹¹⁾ retinal degeneration¹⁶⁾ and heart failure.¹⁷⁾

We found MK as a product of a gene, whose expression was induced at the early stages of the retinoic acid-induced differentiation of teratocarcinoma stem cells.¹⁸⁾ PTN was found as a protein with neurite-promoting activity¹⁹⁾ or as a factor with growth-promoting activity to fibroblasts.²⁰⁾ We reported full protein sequence of MK in 1990,²¹⁾ and the sequence of PTN was reported subsequently^{22),23)}: MK and PTN were revealed to have about 50% sequence identity.

The precisely controlled manner of MK expression during embryogenesis²⁴⁾ and unique features of the protein structure²¹⁾ were sufficient to convince us of the importance of the molecule, and we initiated systematic studies. Glycosaminoglycan-recognizing activity of MK²⁵⁾ was an additional factor tempted me to study MK extensively, since my long term

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Abbreviations: ALK: anaplastic lymphoma kinase; LRP: low density lipoprotein receptor-related protein; MFP: medial floor plate; MK: midkine; PTN: pleiotrophin; PTP ζ : Receptor-like protein tyrosine phosphatase- ζ .

Table 1. Actions of MK to cultured cells

Promoted cellular activities	Target cells
<i>Soluble MK</i>	
Growth	Fibroblasts, ³⁶⁾ keratinocytes, ¹⁵¹⁾ tumor cells ^{6),12)}
Survival	Embryonic neurons ^{7),51)-53)}
Contraction of collagen gels	Fibroblasts ¹⁵²⁾
Synthesis of extracellular matrices	Fibroblasts ¹⁵³⁾
Synthesis of cytokines	Endothelial cells, ⁵⁴⁾ renal epithelial cells ⁹⁾
Fibrinolytic activity	Endothelial cells ¹⁵⁴⁾
Migration	Neutrophils ⁵⁵⁾
<i>Substratum-bound MK</i>	
Growth	Neural precursor cells ⁵⁸⁾
Survival	Neural precursor cells ⁵⁸⁾
Extension of neurite or processes	Embryonic neurons, ^{36),37)} oligodendrocyte precursor-like cells ⁶⁶⁾
Migration	Neurons, ⁸⁾ osteoblast-like cells, ⁵⁷⁾ neutrophils, ⁵⁵⁾ macrophages ⁵⁶⁾
Clustering of acetylcholine receptors	Myoblasts ⁸⁵⁾

the chemical synthesis, MK with aberrant disulfide bridges was rigorously removed. MK is an adhesive protein, and care is needed to avoid absorption to vessels as explained elsewhere.⁴¹⁾

The human MK gene (*MDK*) is present on chromosome 11 at p11.2⁴²⁾ between the diacylglycerol kinase α gene and muscarinic acetylcholine receptor 4 gene,¹⁾ while the mouse counterpart (*Mdk*) is on chromosome 2.⁴³⁾ *MDK* and *Mdk* encompass 2 kb and have 4 exons.^{44),45)}

MK expression is induced by retinoic acid, and the promoter region has a functional retinoic acid-responsive element.⁴⁶⁾ Glucocorticoid suppresses MK expression through its nuclear receptor.⁴⁷⁾ The promoter region also has a binding site for WT-1, the product of the Wilms' tumor suppressor gene.⁴⁸⁾ Furthermore, hypoxia induces MK expression through the binding of hypoxia inducible factor-1 α (HIF-1 α) to a hypoxia responsive element in the MK promoter.⁴⁹⁾ MK expression is strictly controlled both spatially and temporally during embryogenesis.^{24),50)} Generally, MK is most intensely expressed during midgestation, while the expression is weak or absent in the majority of adult tissues.^{1),24)}

***In vitro* activities and mechanism of action**

MK exhibits various activities *in vitro* (Table 1). For example, soluble MK promotes the growth of fibroblasts,³⁶⁾ survival of embryonic neurons^{1),7),51)-53)} and expression of chemokines.^{9),54)} The substratum-bound

form enhances outgrowth of neurites^{6),36),37)} and migration of neutrophils,⁵⁵⁾ macrophages,⁵⁶⁾ embryonic neurons⁸⁾ and osteoblast-like cells.⁵⁷⁾ Occasionally, substratum-bound MK shows stronger activity than the soluble form. For example, the growth and survival of neural precursor cells is promoted only by substratum-bound MK.⁵⁸⁾ Probably, MK in the tissue is delivered to target cells as a matrix-bound form. Inhibition of MK-dependent migration of UMR106 osteoblast-like cells is frequently employed in the screening of MK inhibitors (Matsui *et al.*, unpublished observations).

MK is inhibited by heparin, a sulfated glycosaminoglycan. Therefore it is likely that recognition of glycosaminoglycans in proteoglycans is essential for MK activities. Oligomers of heparan sulfate trisulfated units and those of chondroitin sulfate E units have been identified as structures required for strong binding to MK^{41),59)-61)} (Fig. 2). The affinity of the two structures for MK is considered similar, based on behavior upon MK-affinity chromatography, surface plasmon resonance spectroscopy, and inhibition of neurite outgrowth.⁴¹⁾ The presence of these high-affinity binding structures in the embryonic brain of mice has been confirmed by an analysis of glycosaminoglycans synthesized by the cells.⁶¹⁾

Receptor-like protein tyrosine phosphatase- ζ (PTP ζ),⁸⁾ syndecans,^{50),62),63)} glypican-2,⁶⁴⁾ PG-M/versican⁶⁵⁾ and neuroglycan C⁶⁶⁾ are proteoglycans with strong affinity for MK. Among them, PTP ζ , a

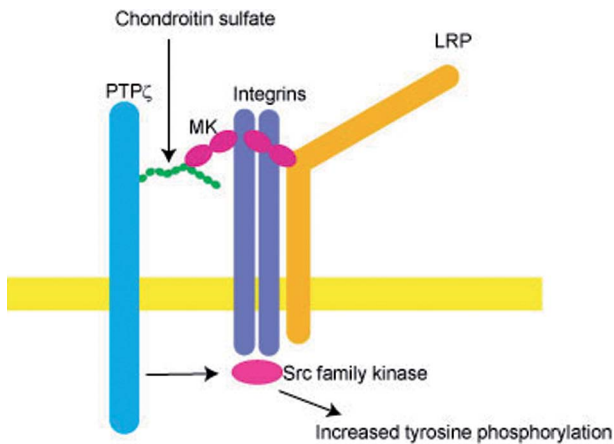


Fig. 3. A proposed model of MK action. MK, which acts as a dimer, binds to components of the receptor including glycosaminoglycan chains, and promotes the formation of the receptor complex. When an MK dimer binds to an integrin and the chondroitin sulfate chain of PTP ζ , the cytoplasmic phosphatase domain of PTP ζ becomes closer to the cytoplasmic domain of the integrin, resulting in an increase in the tyrosine phosphorylation of key signaling molecules.

chondroitin sulfate proteoglycan, is an established component of the MK receptor. PTP ζ binds to MK with a Kd of 0.56 nM, which decreases to 8.8 nM after the removal of chondroitin sulfate chains.⁸⁾

Analyses of MK-binding proteins from embryonic brains have revealed that low density lipoprotein receptor-related protein (LRP),⁶⁷⁾ $\alpha_4\beta_1$ -integrin and $\alpha_6\beta_1$ -integrin⁶⁸⁾ also serve as MK receptors. These proteins and PTP ζ form a receptor complex, and MK promotes this process.⁶⁸⁾ A model of MK action through the receptor complex is shown in Fig. 3. By the complex formation, PTP ζ and the downstream signaling systems of integrins might come to be located close together.

After MK stimulation, intracellular tyrosine phosphorylation increases. Inhibitors of Src family kinases hinder MK activities, indicating the importance of these kinases in signaling.⁵⁷⁾ After MK action, PTP ζ may act on a tyrosine phosphate residue, which locks Src kinase in an inactive state. Removal of the phosphate residue will lead to the activation of Src, as in the case of PTN signaling.⁶⁹⁾ Alternatively, MK stimulates dimerization of PTP ζ , leading to inactivation of PTP ζ , which might compete with Src.⁷⁰⁾ An observation that vanadate, an inhibitor of PTP ζ , inhibits MK⁵⁷⁾ appears to favor the former view.

Increased tyrosine phosphorylation of paxillin accompanying MK action occurs in case of cell mi-

gration of osteoblast-like cells.⁶⁸⁾ Upon promotion of survival, activation of PI3 kinase and MAP kinase takes place, followed by suppression of caspases.^{7),71)}

For promotion of the migration and invasion of human head and neck squamous cell carcinoma cells, MK binds to $\alpha_6\beta_1$ -integrin and tetraspanin, and induces tyrosine phosphorylation of FAK followed by activation of paxillin and STAT1 α pathway.⁷²⁾ Phosphorylation of STAT3 by MK stimulates the proliferation of postconfluent 3T3-L1 cells, leading to adipogenesis.⁷³⁾ Notch2 is a receptor for MK upon epithelial-mesenchymal transition in immortalized keratinocytes, acting through the Jak2/STAT3 system.⁷⁴⁾ On the other hand, STAT5 phosphorylation is suppressed by MK.¹⁴⁾

Anaplastic lymphoma kinase (ALK) has been proposed to be an MK receptor.⁷⁵⁾ ALK also forms complex with LRP and integrins, suggesting that it is recruited to the receptor complex and plays roles in MK signaling (Muramatsu, H. *et al.*, unpublished observations). After activation by MK, ALK phosphorylates insulin receptor substrate-1, activates MAP kinase and PI3 kinase and causes transcriptional activation of NF κ B.⁷⁶⁾

Neuroglycan C serves as an MK receptor upon process extension in oligodendrocyte precursor-like cells.⁶⁶⁾ Neuroglycan C also functions in a receptor complex (Ichihara *et al.*, unpublished observations). In addition to various cell-surface proteins mentioned above, neuropilin-1 was identified in MK-binding proteins from UMR106 osteoblast-like cells (Muramatsu, H. *et al.*, unpublished observations).

MK binds to nucleolin, a nuclear protein which is also located at the cell surface and functions as a shuttle to the nucleus.³⁹⁾ LRP, a component of the MK receptor, serves to internalize the bound MK.⁷⁷⁾ After this internalization, cytoplasmic MK is transferred to the nucleus by nucleolin⁷⁷⁾ and by laminin-binding protein precursor,⁷⁸⁾ and the transportation is important to cell survival induced by MK.⁷⁷⁾ Thus, MK might also act within the nucleus. Indeed, MK transferred to the nucleolus is involved in the synthesis of ribosomal RNA at the place.⁷⁹⁾ Translation initiation factor (eIF3) is also an MK-binding protein in the embryonic brain, the physiological significance of which remains to be established (Muramatsu, H. *et al.*, unpublished observations).

Neurogenesis

During midgestation period, MK expression is intense in neural tissue, epithelial tissue in the pro-

cess of epithelial-mesenchymal interactions and mesoderm undergoing remodeling.^{24),50)} In each of these tissues, further studies have revealed the developmental significance of MK activity.

During the development of *Xenopus laevis*, MK is found in the neural anlagen and strongly expressed in the brain and spinal cord. When MK mRNA is injected into the dorsal vegetal region of 8-cell embryos, neural tissues enlarge abnormally. The excised ectoderm from this embryo is enhanced in development of anterior neural tissue after activin treatment, and is suppressed in mesoderm induction as compared to the normal ectoderm.⁸⁰⁾

The role of MK in the initial stages of neurogenesis has been elucidated in the zebrafish, which has two MK molecules (Mdka and Mdkb) as a result of gene duplication. Mdka is expressed in the paraxial mesoderm and is involved in the formation of the medial floor plate (MFP) in the adjacent neural tube. MFP organizes the specification of neurons and outgrowth of axons in the ventral spinal cord. Overexpression of Mdka results in an enlargement of the MFP and reduction in the size of the notochord, and downregulation of Mdka expression results in a defective MFP and in increased cell density of the notochord.⁸¹⁾ Mdka appears to promote the growth and survival of MFP progenitors, and the increased number of MFP cells probably explains the reduction in size of the notochord. On the other hand, overexpression and knockdown experiments have indicated that Mdkb expressed in the neural plate is required for the earliest steps of cell specification at the neural plate border and is essential for the development of neural crest cells and sensory neurons.⁸²⁾

In the developing cortex of mice and rats, MK expression is intense in the basal layer, which is composed of proliferating neural precursor cells including neural stem cells.^{24),83)} Radial glial processes are extended structures derived from neural stem cells, and differentiated neurons migrate externally along these processes. MK is also strongly expressed in radial glial processes, and is a good immunohistochemical marker of the processes.^{83),84)} An *in vitro* study has shown that MK enhances the growth and survival of neural precursor cells without inhibiting their differentiation capability.⁵⁸⁾ This finding provides a cell biological basis to MK activities found in *Xenopus laevis*. In addition, MK secreted by the neurites of embryonic neurons induces clustering of

acetylcholine receptors on myoblasts, suggesting that MK is involved in the formation of synapses.⁸⁵⁾

In spite of important roles of MK in neurogenesis, mice deficient in *Mdk* exhibit normal phenotypes in overall neural functions.⁸⁶⁾ However, in depth analysis of the deficient mice revealed deficits in specific neural functions. These mice are in a hypodopaminergic state in terms of levels of dopamine and its receptors, with defects related to dopamine function (prepulse inhibition).⁸⁷⁾ Furthermore, mice deficient in *Mdk* or *Ptn* exhibit a moderate auditory deficit, while mice deficient in both show a severer phenotype.⁸⁸⁾ Although the defect in the single knockout mice may be due to deficit in sensory neurons, the severe defect in the double knockout could principally be caused by abnormalities in the cochlea, including a drastic decrease in β -tectorin expression. In any event, it is likely that the loss of MK is usually compensated for by other molecules during mouse neurogenesis, but in certain regions it is not compensated leading to expression of phenotypes. Detailed analyses of MK deficient mice are expected to reveal more neurological phenotypes.

Epithelial-mesenchymal interactions

The role of MK in epithelial-mesenchymal interaction has been studied using an artificial blood vessel model, in which vascular endothelial cells from the human umbilical cord are cultured on human aortic smooth muscle cells.⁵⁴⁾ The endothelial cells secrete MK, which acts on smooth muscle cells and induces production of factors including IL8. IL8 then acts on the endothelial cells and promotes their growth. Thus, MK plays a key role in the interaction between epithelial cells and mesenchymal cells. In the *in vitro* differentiation system of the lung germ, MK produced by epithelial tissue stimulates the development of mesenchymal tissue.⁸⁹⁾ The development of tooth germ *in vitro* is inhibited by anti-MK antibody.⁹⁰⁾ In this system, MK appears to suppress excessive activity of BMP-2. Furthermore, MK which is expressed in lung epithelial cells causes vascular remodeling in the organ.⁴⁹⁾ MK is also involved in the epithelial-mesenchymal transition of tumor cells.⁷⁴⁾

In terms of the remodeling of mesenchyme, MK has been identified as an autocrine factor that induces adipocyte formation from 3T3-L1 cells.⁷³⁾ Furthermore, transfection of MKcDNA to chondrogenic cells enhances chondrogenesis.⁹¹⁾

Table 2. Overexpression of MK in human tumors

Tumors	General	Relation to prognosis	Target of therapy
Oral squamous cell carcinoma	Ruan <i>et al.</i> 2007 ¹⁵⁵⁾	Ruan <i>et al.</i> 2007 ¹⁵⁵⁾	
Esophageal carcinoma	Aridome <i>et al.</i> 1995 ⁹⁵⁾		
Gastric carcinoma	Aridome <i>et al.</i> 1995 ⁹⁵⁾		
Colon carcinoma	Aridome <i>et al.</i> 1995 ⁹⁵⁾		Takei <i>et al.</i> 2001 ¹²⁾
Hepatocellular carcinoma	Aridome <i>et al.</i> 1995 ⁹⁵⁾		Dai <i>et al.</i> 2009 ¹²⁴⁾
Pancreatic head carcinoma	Maeda <i>et al.</i> 2007 ¹⁵⁶⁾	Maeda <i>et al.</i> 2007 ¹⁵⁶⁾	
Lung carcinoma	Garver <i>et al.</i> 1993 ⁹⁶⁾		
Urinary bladder carcinoma	O'Brien <i>et al.</i> 1996 ⁹⁷⁾	O'Brien <i>et al.</i> 1996 ⁹⁷⁾	
Prostate carcinoma	Konishi <i>et al.</i> 1999 ⁹⁸⁾	Konishi <i>et al.</i> , 1999 ⁹⁸⁾	Takei <i>et al.</i> 2006 ¹²²⁾
Breast carcinoma	Garver <i>et al.</i> 1994 ¹⁵⁷⁾		
Uterine carcinoma	Moon <i>et al.</i> 2003, ¹⁵⁸⁾ Tanabe <i>et al.</i> 2008 ¹⁵⁹⁾	Tanabe <i>et al.</i> 2008 ¹⁵⁹⁾	Tanabe <i>et al.</i> 2008 ¹⁵⁹⁾
Ovarian carcinoma	Nakanishi <i>et al.</i> 1997 ¹⁶⁰⁾		
Osteosarcoma	Maehara <i>et al.</i> 2007 ¹²⁵⁾		Maehara <i>et al.</i> 2007 ¹²⁵⁾
Soft tissue sarcoma	Jin <i>et al.</i> 2008 ¹⁶¹⁾		Jin <i>et al.</i> 2008 ¹⁶¹⁾
Neuroblastoma	Nakagawara <i>et al.</i> 1995 ⁹⁹⁾	Nakagawara <i>et al.</i> 1995 ⁹⁹⁾	
Astrocytoma	Mishima <i>et al.</i> 1997 ¹⁰⁰⁾	Mishima <i>et al.</i> 1997 ¹⁰⁰⁾	
Meningioma	Tong <i>et al.</i> 2007 ¹⁶²⁾		
Childhood B-precursor acute lymphoblastic leukemia	Hidaka <i>et al.</i> 2007 ¹⁶³⁾		
Wilms' tumor	Tsutsui <i>et al.</i> 1993 ⁹⁴⁾		
Malignant peripheral nerve sheath tumor	Mashour <i>et al.</i> 2001 ¹⁰⁵⁾		

Reproduction

In spite of various roles of MK in developmental processes, MK-deficient mice are born without major defects.⁸⁶⁾ The same is true for mice deficient in PTN. However, mice deficient in both were born with 1/3 of the frequency expected by Mendelian segregation,¹⁰⁾ were small in size¹⁰⁾ and about 50% died before 4 weeks (Muramatsu, H. *et al.*, unpublished observations). Thus, MK and PTN play important roles in development, and compensate for each other. Furthermore, the double knockout mice exhibited female infertility: after repeated mating with wild-type males, 79% of the double-deficient females remained sterile.¹⁰⁾ Defects in follicular maturation, an altered estrous cycle and vaginal malformation have been found to be the basis of the infertility.

Among them, the defects in follicular maturation appear to be the principal cause; MK and PTN are concluded to be important in follicular maturation.¹⁰⁾

The efficiency with which *in vitro* fertilized bovine embryos develop to the blastocyst stage is increased by adding MK to the culture medium.⁹²⁾ This effect of MK is mediated by cumulus cells surrounding oocytes. MK acts on cumulus cells to help them survive and secrete factors acting on oocytes.⁹³⁾ This MK activity has practical importance. Furthermore, a similar mechanism can be considered for the actions of MK and PTN to promote follicular maturation.

Cancer

MK is overexpressed in many malignant tumors of humans (Table 2),^{1),94)} including hepatocellular

carcinoma,⁹⁵⁾ gastric carcinoma,⁹⁵⁾ colon carcinoma,⁹⁵⁾ lung carcinoma,⁹⁶⁾ urinary bladder carcinoma,⁹⁷⁾ prostate carcinoma,⁹⁸⁾ neuroblastoma⁹⁹⁾ and astrocytoma.¹⁰⁰⁾ Overexpression is observed in about 80% cases in respective tumors.¹⁾ Furthermore, patients with high MK expression in the tumor frequently have a worse prognosis than those with low MK expression (Table 2).^{1),97)–100)} MK is expected to contribute to tumor invasion by promoting growth, survival and migration of tumors and by promoting angiogenesis. Indeed, transfection with MK cDNA results in malignant transformation of NIH 3T3 cells.¹⁰¹⁾ Furthermore, *MDK* has been identified to be the gene, overexpression of which is most closely correlated with resistance to chemotherapeutics in human gastric cancer cell lines.¹⁰²⁾ MK has been also shown to be a mediator of chemotherapy resistance to neighboring cells.¹⁰³⁾ AcylCoA synthetase 5 is frequently overexpressed in malignant gliomas and contributes to cell survival under extracellular acidosis. Overexpression of the enzyme leads to increased expression of MK, which is partly responsible for the effect of the enzyme.¹⁰⁴⁾

The induction of MK in various tumors is probably mediated by hypoxic conditions in the tumor.⁴⁹⁾ Loss of function of WT1, Wilms' tumor suppressor gene, appears to be the cause of high levels of MK expression in Wilms' tumor,⁹⁴⁾ since a functional WT1 binding site is present in the MK promoter.⁴⁸⁾ Oncogenesis due to loss of function of WT1 is likely to involve the induction of MK expression. Furthermore MK is overexpressed in malignant peripheral nerve sheath tumor, based on a lack of function of NF1, another tumor suppressor gene.¹⁰⁵⁾

Serum MK levels can be measured with a sandwich enzyme-linked immunoassay.^{106),107)} The levels are increased in patients with cancer,^{106)–108)} rheumatoid arthritis¹³⁾ and Alzheimer's disease.¹⁰⁹⁾ Because of the distinct clinical features of these diseases, MK is expected to be helpful in the screening of cancer.^{106),107),110)–113)} MK levels are not increased in the cases of viral hepatitis,¹¹⁰⁾ with a few exceptions, in which hepatocellular carcinoma developed later (Salama *et al.*, unpublished observations). Patients with high MK serum levels had a poor prognosis in cases of esophageal carcinoma¹¹¹⁾ and neuroblastoma.^{114),115)} For large scale screening, a combination of two monoclonal antibodies and usage of beads and an automated system appear to be especially helpful.¹¹⁶⁾ Because of its elevated levels even

early on, irrespective of α -fetoprotein levels, MK is considered to be helpful in detection of α -fetoprotein-negative hepatocellular carcinoma.¹¹²⁾ Further information is provided in a previous review.¹⁰⁸⁾

In terms of cancer detection, a truncated MK in which exon 3 is skipped by alternative splicing may also be helpful.¹¹⁷⁾ In certain carcinomas such as colon carcinoma, a truncated MK protein is expressed only in tumor tissue, especially in metastasized lymph nodes.^{117)–119)} Although the truncated form was initially detected as mRNA, subsequent study revealed the truncated protein by using a specific antibody.¹²⁰⁾ Truncated MK can also transform fibroblasts.¹²¹⁾

The involvement of MK in tumor progression implies that inhibition of the synthesis or action of MK will contribute to cancer therapy. Indeed, an antisense oligoDNA to MK inhibits the growth of mouse colorectal carcinoma cells in culture,¹²⁾ most probably by down-regulating intracellular tyrosine phosphorylation as mentioned in the previous section. Furthermore the antisense oligoDNA suppresses the growth of the tumor in nude mice. The delivery of antisense oligoDNA into pregrown tumors has been achieved with the aid of atellocollagen.¹²⁾ siRNA to human MK enhances the growth inhibitory effect of paclitaxel on human prostate carcinoma cells grown in nude mice.¹²²⁾ It is noted that inhibition of the synthesis of human MK had less of an effect on tumor growth than that of mouse MK. This is probably because even after inhibition of the synthesis of human MK, mouse MK provided by the host supports the growth of the xenografted tumor. Indeed, lung metastasis of an MK-negative tumor is less severe in MK-deficient mice than in wild-type mice.¹²³⁾ The growth of hepatocellular carcinoma in mice is inhibited by antisense oligoDNA to MK delivered by nanoparticles.¹²⁴⁾

Although polyclonal anti-MK antibodies inhibit the growth of tumor cells *in vitro*,^{6),125)} many monoclonal antibodies to MK do not have a strong effect, probably because not only extracellular MK, but also intracellular MK contributes to tumor growth, and only a population of anti-MK is able to inhibit intracellular MK. Aptamers to MK did not exhibit significant growth inhibitory effects to tumor cells either. A promising candidate to inhibit MK is a low molecular weight compound. After *in silico* screening we found that some trifluoro compounds inhibited MK-dependent migration of osteoblast-like cells without significant cytotoxic effects (Muramatsu, T. *et al.*,

unpublished results). Peptide fragments of MK receptors such as LRP¹²⁶⁾ and integrins (Muramatsu, T. *et al.*, unpublished observations) are also promising. siRNA and antisense oligoDNA to MK have already demonstrated potent inhibitory effects on tumor growth *in vivo*. Once delivery methods to distant metastatic nests are developed, these agents will become efficient therapeutics to inhibit tumor invasion.

Preferential expression of toxic genes in cancer cells with the help of the MK promoter is also promising as an aid in cancer therapy.^{127)–130)} The MK promoter fused with thymidine kinase gene is especially helpful in adenovirus-mediated gene transfer followed by ganciclovir treatment.¹²⁸⁾ Since the MK promoter cannot act in the liver, its use overcomes one major problem in the regime, namely liver toxicity.

Inflammation and repair

MK expression is induced in damaged tissues, especially after ischemia in blood vessels,⁵⁶⁾ the brain cortex¹³¹⁾ and the myocardium,¹⁷⁾ and exhibits two effects, an enhancement of inflammation and a promotion of survival and repair. Thus, MK is either beneficial or harmful to the injured tissue, depending on its origin. Phenotypes of MK-deficient mice have revealed the role of MK in a pathological state. As an example, upon partial hepatectomy, the remaining liver of MK-deficient mice exhibits less inflammation than that of wild-type mice. However, the growth of the liver is also less extensive in the deficient mice, and as a whole MK-deficiency hinders liver regeneration.¹³²⁾

MK enhances inflammation by promoting the migration of inflammatory leukocytes,^{9),55),56)} inducing synthesis of chemokines⁹⁾ and suppressing the increase of regulatory T cells.¹⁴⁾ Upon ischemic injury to blood vessels, MK recruits inflammatory leukocytes, which secrete factors promoting the migration of smooth muscle cells and trigger the formation of neointima.⁵⁶⁾ This neointima formation, which is a model of restenosis after balloon therapy of infarcted coronary vessels, is less extensive in MK-deficient mice than in wild-type mice.⁵⁶⁾ MK deficiency also has beneficial effects on antibody-induced arthritis (a model of rheumatoid arthritis),¹³⁾ experimental autoimmune encephalitis (a model of multiple sclerosis),¹⁴⁾ adhesion after surgery¹³³⁾ and nephritis, which is caused either by ischemia,⁹⁾ exposure to chemo-

therapeutics¹³⁴⁾ or diabetes.¹³⁵⁾ The MK-deficient mice used in these experiments were repeatedly backcrossed to C57BL/6 mice so that both mice were in a syngenic state. That the genes flanking *Mdk* were not altered in the deficient mice was confirmed by sequencing (unpublished observations).

In the above-mentioned diseases, MK inhibitors are candidates for therapeutics. siRNAs, antisense oligoDNAs and aptamers to MK have already proved effective in experimental models (Table 3). Notable examples are the treatment of nephritis with an antisense oligoDNA to MK,¹³⁶⁾ of neointima formation with an siRNA to MK¹³⁷⁾ and of experimental autoimmune encephalitis using an RNA aptamer to MK.¹⁴⁾ MK inhibitors might be also effective in the treatment of endometriosis, because of the possible involvement of MK in the disease.¹³⁸⁾

MK is a regulator of the renin-angiotensin system.^{15),139)} Hypertension is induced by 5/6 nephrectomy in wild-type mice, but not significantly in MK-deficient mice.¹⁵⁾ Further studies have revealed that MK is expressed in the lung after 5/6 nephrectomy, and induces the expression of angiotensin-converting enzyme in microvascular endothelial cells.¹⁵⁾ MK appears to be a key molecule causing hypertension upon chronic nephritis. Thus, there is a possibility that MK inhibitors become useful for the treatment of hypertension. MK is also known to suppress catecholamine biosynthesis in the aorta, but not in other tissues.¹⁴⁰⁾

The role of MK in the reparative process was first demonstrated in the retina. Light-induced retinal degeneration is suppressed by the injection of MK into the subretinal region.¹⁶⁾ Injection of MK into the brain ventricle delays the onset of the death of hippocampal neurons after ischemia.¹¹⁾ Transfection and expression of MK is also effective.¹⁴¹⁾ Both degeneration and regeneration of injured peripheral nerve are delayed in *Mdk*-deficient mice compared to wild-type mice.¹⁴²⁾ After ischemia and reperfusion of the heart, more *Mdk*-deficient mice die due to heart failure than wild-type mice.¹⁷⁾ Delivery of MK into the heart substantially enhances the survival of the mice.¹⁷⁾ MK also improves heart function upon chronic heart failure after ischemic injury.^{143),144)} Thus, in these diseases, MK itself is expected to be a potent therapeutic by promoting survival of damaged tissues (Table 3). MK produced by the yeast system is best suited for this purpose, since large scale production is possible and the yield of

Table 3. MK as a target of therapeutics or a therapeutic

Diseases and abnormalities	Phenotypes of MK KO mice	Animal experiments using therapeutics
Restenosis	Horiba <i>et al.</i> 2000 ⁵⁶⁾	Banno <i>et al.</i> 2006 ¹³⁷⁾ (siRNA), Hayashi <i>et al.</i> 2005 ¹⁶⁴⁾ (AS oligoDNA)
Rheumatoid arthritis	Maruyama <i>et al.</i> 2004 ¹³³⁾	Yamamoto <i>et al.</i> 2006 ¹⁶⁵⁾ (CSE, siRNA)
Multiple sclerosis	Wang <i>et al.</i> 2008 ¹⁴⁾	Wang <i>et al.</i> 2008 ¹⁴⁾ (RNA aptamer)
Adhesion after surgery	Inoh <i>et al.</i> 2004 ¹³³⁾	Yamamoto <i>et al.</i> 2006 ¹⁶⁵⁾ (siRNA)
Nephritis	Sato <i>et al.</i> 2001, ⁹⁾ Kawai <i>et al.</i> 2004, ¹³⁴⁾ Kosugi <i>et al.</i> 2006 ¹³⁵⁾	Sato <i>et al.</i> 2005 ¹³⁶⁾ (AS oligoDNA)
Hypertension	Hobo <i>et al.</i> 2009 ¹⁵⁾	
Malignant tumors	Salama <i>et al.</i> 2006 ¹²³⁾	Takei <i>et al.</i> 2001 ¹²⁾ (AS oligoDNA), Takei <i>et al.</i> 2006 ¹²²⁾ (siRNA), Dai <i>et al.</i> 2009 ¹²⁴⁾ (AS oligoDNA)
Neuronal death	Sakakima <i>et al.</i> 2009 ¹⁴²⁾	Yoshida <i>et al.</i> 2001 ¹¹⁾ (MK)
Retinal degeneration		Unoki <i>et al.</i> 1994 ¹⁶⁾ (MK)
Heart failure	Horiba <i>et al.</i> 2006 ¹⁷⁾	Horiba <i>et al.</i> 2006 ¹⁷⁾ (MK) Fukui <i>et al.</i> 2008 ¹⁴³⁾ (MK), Takenaka <i>et al.</i> 2009 ¹⁴⁴⁾ (MK)

Either an MK inhibitor or MK itself used as therapeutics was shown in the parenthesis. Disease was severer in MK KO mice when MK was used as therapeutics, and less severe when an MK inhibitor was used as therapeutics. AS, antisense; CSE, chondroitin sulfate E.

properly folded protein is high compared to production in bacteria.

MK is expressed in senile plaques in the brain of the patients.¹⁴⁵⁾ Senile plaques are hallmark of the disease, and the primary deposit is amyloid β -peptide. In the brain of aged MK-deficient mice, plaque formation of amyloid β -peptide is more than in the brain of age-matched wild-type mice. Thus, MK expression in Alzheimer's disease might be induced to counteract the progression of the disease (Muramatsu, H. *et al.*, unpublished observations). Indeed, MK inhibits cytotoxicity of amyloid β -peptide and its fibril formation.^{146,147)} MK expression is also markedly increased in the prefrontal cortex of chronic alcoholics, concomitant with shrinkage of the neural tissue.¹⁴⁸⁾ MK is probably synthesized to counteract the loss of neurons also in this case. In the rat hippocampus, chronic administration of morphine and yohimbine also upregulates MK expression.¹⁴⁹⁾

Conclusions and perspectives

MK is involved in a variety of physiological and pathological processes. I expect that significant roles of MK will be further revealed even in fields not mentioned in this review. In this context, it is proper to

mention that MK may be involved in protection against HIV infection, since the binding of HIV to nucleolin appears to be required for viral entry, and MK competes with HIV for nucleolin binding.¹⁵⁰⁾

Our studies have shown that the MK receptor is a molecular complex containing proteoglycans. More studies are required to clarify all the components of the receptor complex and their mutual interactions. Essential roles of the MK family in development and reproduction have been established by severe phenotypes of mice doubly deficient in both MK and PTN. In depth analysis of MK single-knockout mice is expected to reveal new functions of MK, unshared with PTN.

Clinical application of MK inhibitors is much needed, especially for treatment of malignancy. The improvement in delivery methods of oligonucleotide reagents and development of low molecular weight inhibitors are among the subjects on which further basic research is required for the purpose.

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Profile

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