Expression of the heparin-binding cytokines, midkine (MK) and HB-GAM (pleiotrophin) is associated with epithelial-mesenchymal interactions during fetal development and organogenesis

Thimios A. Mitsiadis1,*, Markku Salmivirta2,3, Takashi Muramatsu4, Hisako Muramatsu4, Heikki Rauvala5, Eero Lehtonen6, Markku Jalkanen3 and Irma Thesleff1

1Institute of Dentistry, Department of Pedodontics and Orthodontics, University of Helsinki, Helsinki, Finland
2Department of Medical Biochemistry, University of Turku, Turku, Finland
3Turku Centre for Biotechnology, University of Turku, Turku, Finland
4Department of Biochemistry, School of Medicine, Nagoya University, Nagoya, Japan
5Institute of Biotechnology, University of Helsinki, Helsinki, Finland
6Department of Pathology, University of Helsinki, Helsinki, Finland

*Author for correspondence (present address): T. Mitsiadis, Department of Cell and Molecular Biology, Karolinska Institute, 171 77 Stockholm, Sweden

SUMMARY

Midkine (MK) and heparin binding-growth associated molecule (HB-GAM or pleiotrophin), constitute a new family of heparin-binding proteins implicated in the regulation of growth and differentiation (T. Muramatsu (1993) Int. J. Dev. Biol. 37, 183-188). We used affinity-purified antibodies against MK and HB-GAM to analyze their distribution during mouse embryonic development. From 9 to 14.5 day post-coitum (dpc), both proteins were detected in central and peripheral nervous systems, facial processes, limb buds, sense organs, respiratory, digestive, urogenital, and skeletal systems. MK and HB-GAM were often localized on the surface of differentiating cells and in basement membranes of organs undergoing epithelial-mesenchymal interactions. The levels of MK protein decreased considerably in the 16.5 dpc embryo, whereas HB-GAM staining persisted in many tissues. Our in situ hybridization results revealed a widespread expression of MK transcripts that was not always consistent with the distribution of MK protein in developing tissues. In many epithelio-mesenchymal organs MK and HB-GAM were codistributed with syndecan-1, a cell surface proteoglycan. In limb buds and facial processes, MK, HB-GAM, and syndecan-1 were localized to the apical epithelium and the adjacent proliferating mesenchyme. Both MK and HB-GAM bound syndecan-1 in solid-phase assays in a heparan sulfate-dependent manner. The biological effects of MK and HB-GAM on limb and facial mesenchyme were studied in vitro by application of beads preloaded with the proteins. Neither MK nor HB-GAM stimulated mesenchymal cell proliferation or induced syndecan-1 expression. Taken together these results indicate that MK and HB-GAM may play regulatory roles in differentiation and morphogenesis of the vertebrate embryo, particularly in epithelio-mesenchymal organs, and suggest molecular interactions with syndecan-1.

Key words: midkine, pleiotrophin, HB-GAM, syndecan-1, retinoic acid, embryonic development, epithelial-mesenchymal interactions

INTRODUCTION

Classical studies of experimental embryology demonstrated that inductive interactions between epithelial and mesenchymal tissues lead to the determination of cell fate and the subsequent formation of tissues and organs (Gurdon, 1992; Saxén, 1972; Saxén et al., 1980). The molecular changes associated with tissue interactions have been analyzed during recent years, and regulatory roles have been proposed for growth factors, extracellular matrix molecules and various cell surface components (Bernfield et al., 1992; Chiquet-Ehrismann et al., 1986; Jalkanen et al., 1993; Thesleff et al., 1991; Vainio et al., 1989, 1993). Heparin-binding growth factors are expressed in developing organs (Gonzalez et al., 1990; Niswander and Martin, 1992; Wilkinson et al., 1988) and they appear to have signaling roles in embryonic induction (Niswander et al., 1993).

Midkine (MK) is a heparin-binding molecule that can be induced in some cell types by treatment with retinoic acid (RA) (Huang et al., 1990; Kadomatsu et al., 1988; Matsubara et al., 1990). This $13 \times 10^3$ M<sub>r</sub> secreted protein is unusually rich in cysteine and basic amino acids, and promotes the growth of neuronal and PC12 cells (Kadomatsu et al., 1988; Michikawa et al., 1993; Muramatsu, 1993; Tomomura et al., 1990). Another heparin-binding protein with a molecular mass of about $18 \times 10^3$, the heparin binding-growth associated molecule (HB-GAM), has been isolated from perinatal rat brain (Rauvala, 1989). HB-GAM has 50% sequence identity with...
MK (Merremmies and Rauvala, 1990), exhibits neurite outgrowth activity (Hampton et al., 1992; Raualvo et al., 1992; Rauvala, 1989), and may be a mitogen for some cell types (Li et al., 1990). Because of the suspected widespread role of HB-GAM the term pleiotropin has been proposed (Li et al., 1990). RIHB (retinoic acid-induced heparin-binding protein) is another protein isolated from the chicken embryo (Vigny et al., 1989). RIHB has 65% sequence identity with MK (Urios et al., 1991), exhibits mitogenic activity and enhances neurite outgrowth in PC12 cells (Raulais et al., 1991). Because of RA-induced expression, it has been proposed that RIHB is the chick counterpart of MK. All members of this new family of heparin-binding proteins have entirely distinct sequences from fibroblast growth factors (FGFs).

In situ hybridization, slot blot analysis, and immunohistochemical studies have demonstrated that MK, HB-GAM and RIHB are expressed in a variety of tissues during the embryonic development of mouse (Kadomatsu et al., 1990; Muramatsu et al., 1993; Nakamoto et al., 1992), rat (Rauvala et al., 1994; Vanderwinden et al., 1992), and chicken (Duprez et al., 1993; Vigny et al., 1989), suggesting developmental functions for these molecules. Functional studies with purified MK, HB-GAM and RIHB proteins have reported a wide range of effects on several cell lines including teratocarcinoma, leukemia, and PC12 cells (Hampton et al., 1992; Li et al., 1990; Maruta et al., 1993; Muramatsu et al., 1993; Nurcombe et al., 1992; Raulais et al. 1991; Raulo et al., 1992; Rauvala, 1989). We decided to analyze the roles of MK and HB-GAM in development, by following their distribution during embryonic mouse development (9-16.5 dpc) by immunohistochemistry. To address the biological roles of MK and HB-GAM in organ development, the proteins were incorporated into beads and applied to limb and jaw mesenchyme in vitro.

Syndecans are a family of cell surface heparan sulfate proteoglycans (HSPG) regulating the biological effects of several heparin-binding molecules (for recent reviews, see Bernfield et al., 1992; Jalkanen et al., 1993; Rapraeger, 1993). Syndecan-1 binds growth factors and several extracellular matrix molecules, such as type I collagen, fibronectin and tenascin (Jalkanen et al., 1993; Salminvira et al., 1991, 1992). Furthermore, the expression of syndecan-1 is regulated by epithelial-mesenchymal interactions during the development of various organs (Solursh et al., 1990; Trautman et al., 1991; Vainio et al., 1989). We compared the distribution of MK and HB-GAM to syndecan-1 and studied the binding of these proteins to syndecan-1.

MATERIALS AND METHODS

Tissue preparation

Hybrid mice (CBAxC57BL and CBAxNMRI) were used at embryonic stages. Embryonic age was determined from the first appearance of the vaginal plug (0 dpc) and by morphological criteria. 9-16.5 dpc mouse embryos were fixed overnight in 4% paraformaldehyde (PFA) in PBS, pH 7.4. The whole embryos were then dehydrated and embedded in paraffin wax. 7 μm serial sections were mounted on silanized slides, dried overnight, and stored in air-tight boxes at 4°C.

Antibodies

MK, HB-GAM, and syndecan-1 antibodies were used for immunohistochemistry. Preparation and characterization of affinity purified rabbit anti-mouse MK polyclonal antibody (aMK), rabbit anti-rat HB-GAM polyclonal antibody (aHB-GAM), and rat anti-mouse syndecan monoclonal antibody 281-2 have been described earlier (Jalkanen et al., 1985; Muramatsu et al., 1993; Rualvo, 1989). Specific binding of aMK (Muramatsu et al., 1993) and aHB-GAM (Rauvala, 1989) has been previously demonstrated using crude extracts of tissues. To confirm that the antibodies do not cross-react, purified MK and HB-GAM proteins were analyzed by western blotting using both antibodies.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Mitsiadis et al., 1992). Briefly, sections were incubated overnight at 4°C with aMK (concentrations 3.3-4 μg/ml), aHB-GAM (concentrations 1.2-2 μg/ml), and rat monoclonal antibody 281-2 against the core protein of syndecan-1 (concentrations 0.75-1 μg/ml) in 0.2% bovine serum albumin (BSA)/2% normal goat serum (NGS)/phosphate-buffered saline (PBS), pH 7.4. Control sections were incubated either with 2% BSA/PBS or with normal rabbit serum. Sections were then incubated with either biotinylated secondary goat anti-rabbit antibody (dilution 1:250 in PBS) or avidin-biotin-peroxidase complex (Vector Laboratories Inc., Burlingame, USA). Peroxidase was revealed by incubation with 3-aminio-9-ethylcarbazole (AEC) containing 1% H2O2.

Heparitinase treatment. Some sections were incubated with 2 μg/ml heparitinase in NaCl/Fi containing 1 mM CaCO3 for 1 hour at 37°C. After several washes the immunological localization of MK was performed as described above.

Probes and in situ hybridization

For in situ hybridization studies, a 629 bp fragment of MK mouse cDNA was subcloned into pGEM3 plasmid. 35S-UTP-labeled (1,000 Ci/mol, Amersham) single-stranded sense (pSP64) and antisense (pSP65) RNA probes were prepared by standard procedures. The pSP64 and pSP65 plasmid vectors were linearized with EcoRI and HindIII restriction enzymes respectively (Promega). The labeled probes were ethanol-precipitated, resuspended in 100 mM DTT, diluted in hybridization solution (60% deionized formamide, 20 mM Tris-HCl, 5 mM EDTA, pH 8, 0.3 M NaCl, 1 x Denhardt’s, 0.5 mg/ml yeast RNA, 10% dextran sulfate), and used at 50,000-60,000 cpm/μl.

In situ hybridization was performed according to the method of Wilkinson and Green (1990). Autoradiography was performed by dipping the slides in autoradiographic emulsion (NTB2 Kodak), and exposing them for 10 days at 4°C. Exposed slides were developed in D-19 (Kodak).

Syndecan-1 binding assay

Metabolically 35SO4-labeled syndecan-1 ectodomain (the extracellular part of the syndecan-1) was isolated from mouse mammary epithelial (NmuMG) cells as previously described (Salminvira et al., 1991). Bovine type I collagen (Boehringer), recombiant murine MK (Muramatsu and Muramatsu, 1991) and rat HB-GAM (Rauulvo, 1992) were used in binding studies. Blot-quality BSA was used as a negative control.

To assess syndecan-1 binding to proteins, equimolar amounts of type I collagen, MK and HB-GAM were incorporated into beads and applied to limb and jaw mesenchyme in vitro.

In order to study the binding mechanism, 50 μg/ml of free heparan sulfate (HS, Sigma) or chondroitin sulfate (CS, Sigma) was added to PBS containing 35SO4-syndecan-1. Dried membranes were covered with plastic foil and the membrane-bound radioactivity was detected using a CS-250 Molecular Imager phosphor imaging and...
high sensitivity imaging screens (BioRad, Hercules, CA). The results were quantified using PhosphorAnalyst software and are expressed as percentages of syndecan-1 bound to MK or HB-GAM in comparison to radioactivity bound to an equimolar amount of type I collagen.

**BrdU labeling and immunostaining of explants as whole mounts**

The distal parts of the forming hindlimb and facial processes of 11-12 dpc mouse embryos were dissected and incubated for 3 minutes in 2.25% trypsin/0.75% pancreatin on ice. Epithelial and mesenchymal tissues were separated under a stereomicroscope. Facial and limb epithelia were recombined to mesenchyme of the same tissue and embryonic age. The tissues were placed on pieces of Nuclepore filters (pore size, 0.1 µm), supported by metal grids (Trowell-type). Affi-gel blue agarose beads (100-200 mesh, 75-150 µm diameter; Bio-Rad) preloaded with MK or HB-GAM protein (diluted into 0.1% BSA to concentrations of 5-100 ng/µl per 5 µl per 50 beads) were then placed on top of the mesenchyme (according to Vainio et al., 1993). Control beads were incubated in 0.1% BSA in PBS. The recombiantins were cultured for 16-32 hours in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS; Gibco) in a humidified atmosphere of 5% CO₂ in air at 37°C. Explants were labeled for 1-2 hours with bromodeoxyuridine (BrdU) according to the manufacturer’s instructions (Amersham and Boehringer Mannheim) and fixed in 4% PFA overnight at 4°C. Explants were incubated with mouse anti-BrdU monoclonal antibody for 1 hour at 37°C, then with biotinylated goat anti-mouse IgG diluted into 10% Seraclone (Jackson Labs, USA) for 30 minutes at 37°C, and finally with avidin-biotin-peroxidase complex (Vector, USA). An antibody against syndecan-1 was also used in whole-mount staining.

**RESULTS**

**Comparative analysis of MK and HB-GAM protein distribution**

Our western-blot analysis demonstrated that the anti-MK antibody reacts only with the MK protein, and that the HB-GAM protein is recognized only by the anti-HB-GAM antibody (Fig. 1). A pattern of widespread yet specific MK and HB-GAM staining was observed in embryonic mouse tissues at 9-10 dpc (data not shown). Immunoreactivity was associated with ectodermal structures, mesoderm- and neuroectoderm-derived cells, and their extracellular matrices. The strongest HB-GAM staining was found in the developing heart. From 12.5 to 14.5 dpc, the MK and HB-GAM staining became more restricted and was detected in ectodermal structures, neuronal tissues, sense organs, facial and limb processes, internal organs, and skeletal tissues (see Table 1 and Figs 2A,B, 3, 4, 5).

**Table 1. Immunocalization of MK, HB-GAM, and syndecan-1 in 14.5 dpc mouse embryos**

<table>
<thead>
<tr>
<th></th>
<th>MK</th>
<th>HB-GAM</th>
<th>Syn-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whisker follicles</td>
<td>ep***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Nose (olfactory)</td>
<td>mes****</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>Inner ear</td>
<td>ep***</td>
<td>**</td>
<td>-</td>
</tr>
<tr>
<td>Eye</td>
<td>mes***</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>Salivary glands</td>
<td>mes***</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>Lung</td>
<td>mes**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Esophagus</td>
<td>mes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stomach</td>
<td>mes**</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>Intestine</td>
<td>mes***</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>mes**</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>Ureter</td>
<td>mes**</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>Pancreas</td>
<td>mes*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Heart</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>Liver</td>
<td>*</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>Testis</td>
<td>*</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>*</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Central nervous system</td>
<td>***</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Neopallial cortex</td>
<td>**</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Midbrain</td>
<td>**</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Diencephalon</td>
<td>**</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Choroid plexus</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>Neural tube</td>
<td>*</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Peripheral nervous system</td>
<td></td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Dorsal root ganglia</td>
<td></td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Neuronal projections (tail)</td>
<td>*</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Dermal epithelium</td>
<td>***</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Oral epithelium</td>
<td>*</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Tongue epithelium</td>
<td>***</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Facial mesenchyme</td>
<td>***</td>
<td>-</td>
<td>***</td>
</tr>
<tr>
<td>Digital mesenchyme</td>
<td>***</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>Cartilage</td>
<td>***</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>Immature bone</td>
<td>***</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>Osteoblasts/osteocytes</td>
<td></td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Muscles</td>
<td>***</td>
<td>***</td>
<td>-</td>
</tr>
<tr>
<td>Endothelia</td>
<td>*</td>
<td>***</td>
<td></td>
</tr>
</tbody>
</table>

(−) no staining; (*) weak staining; (**) moderate staining; (***) strong staining; (NS) not studied. ep, epithelium; mes, mesenchyme; musc, musculature.
5). At 16.5 dpc, MK protein distribution decreased considerably in many developing organs, whereas the HB-GAM staining persisted in a wide variety of tissues. Heparinase treatment of sections reduced or completely abolished the MK staining (data not shown), suggesting that the protein is bound to the extracellular matrix and the cell surfaces through heparan sulfate chains of proteoglycans.

Nervous system
MK and HB-GAM proteins were localized in the neural epithelium from the earliest stage analyzed (9-10 dpc; data not shown). MK was widely expressed in the brain vesicles and in the dorsal part of the neural tube, whereas the distribution of HB-GAM was restricted in some areas of the brain and neural tube. Ganglia and neuronal projections were positive only for HB-GAM. From 12.5 to 14.5 dpc, both proteins were present in the cerebral hemispheres, telencephalon, diencephalon and corpus striatum (Figs 2A,B, 3B, 4A). HB-GAM staining was also observed in the motor nuclei of the trigeminal and facial nerves. Intense expression of MK and HB-GAM was found in the roof of the neopallial cortex, in the intermediate and ventricular zone, whereas only MK staining was observed in the anuclear layer. The meninges were intensely stained for both molecules (Fig. 5Ca,Cb). Intense MK and HB-GAM reactivity was found in the roof of the midbrain and in the olfactory lobe, whereas in the medulla oblongata only some cells were positively stained. In the choroid plexus and the cerebral primordium a very weak staining was observed only for HB-GAM. In the neural tube, MK was restricted to one part of the dorsal area, whereas the HB-GAM staining was diffuse. A nuclear HB-GAM staining was occasionally found in cells situated in the ventral part of the tube (Fig. 4F). HB-GAM was widely distributed in the peripheral nervous system (Figs 4B,C,E, 5Jb), whereas MK staining was evident in the dorsal roots of the ganglia and in some peripheral neurons (Figs 3I, 7Ea). At 16.5 dpc, MK and HB-GAM staining was faint and found in restricted areas of the developing brain (e.g. cerebral primordium for MK), whereas strong HB-GAM reactivity was found in the peripheral nervous system.

Cardiovascular system
At 9 dpc, strong HB-GAM reactivity was found in the developing heart, whereas the MK staining was moderate. From 12.5 to 14.5 dpc, only the valves showed MK reactivity, whereas HB-GAM reactivity was widely distributed in the musculature of the ventricle and the atrium (Fig. 2B). In blood
vessels, a weak MK and HB-GAM staining was observed in endothelial cells and basement membranes (Fig. 7Da). At 16.5 dpc, only HB-GAM protein was localized in heart tissue. At this developmental stage, a strong MK and HB-GAM reactivity was detected in single cells in the dermis (Fig. 3K), presumably macrophages.

**Skeletal system**

At 12.5 dpc, MK protein was detected in precartilaginous mesenchymal condensations of the otic and nose capsule, in the sclerotome-derived precartilage (prevertebrae; Fig. 3C), and in Meckel’s cartilage. At 14.5 dpc, the skeletal elements consist of cartilage, the first ossification centers appear, and the musculature is differentiating. MK and HB-GAM staining was localized in cells involved in endochondral ossification and in differentiating muscles (Figs 2A,B, 5Aa,b). The perichondrium and the hypertrophied chondrocytes were intensely stained for MK (Figs 3D,E, 5Da). HB-GAM immunoreactivity was absent from perichondrium (Fig. 5Db). In the developing sternum, the cartilage was positive only for HB-GAM. Intramembranous ossification implies the direct conversion of mesenchymal cells into osteoblasts. Cells involved in this process forming mandibular, maxillary and cranial bones, exhibited MK and HB-GAM staining (Figs 2A,B, 4A, 5Ca,b). Differentiated osteoblasts and osteocytes were positive only for HB-GAM (Fig. 5Ba,b). MK and HB-GAM appeared to be present in immature bone matrix, whereas mature bone was
Fig. 5. Comparison of the distribution of MK (Xa) and HB-GAM (Xb) in different tissues of 13–16.5 dpc mouse embryos.
(A) Striated muscles at 14.5 dpc.
(B) Maxillary bone at 14.5 dpc.
(C) Meninges and forming cranial bone at 14.5 dpc.
(D) Cartilage of the ribs at 14.5 dpc.
(E) Liver at 14.5 dpc.
(F) Lung at 13 dpc.
(G) Kidney at 13 dpc.
(H) Intestine at 14.5 dpc.
(I) Adrenal gland at 14.5 dpc.
devoid of these proteins. At 16.5 dpc, MK and HB-GAM staining persisted in chondrocytes of several cartilaginous structures, whereas in osteoblasts and muscles only HB-GAM reactivity was found.

Organs and tissues undergoing epithelial-mesenchymal interactions

Sense organs
In the developing eye, MK staining was detected in the neural retina, in lens, and in the condensed mesenchyme around the eye cup (data not shown). In the developing inner ear (12.5-14.5 dpc), MK and HB-GAM staining was observed in the thickened part of the vestibular epithelium, responsible for the sensory function, in the surrounding mesenchyme, and in nerve fibers (Figs 2A,B, 3F, 4I). At 16.5 dpc, a very faint MK staining was observed in sensory epithelium, whereas HB-GAM protein was present in mesenchyme (data not shown). In the developing olfactory organ (from 12.5 to 13.5 dpc), the MK and HB-GAM proteins were distributed in the olfactory epithelium (containing the sensory receptor cells) and the surrounding mesenchyme (Fig. 3I). In its respiratory part, the staining was weaker in epithelium and absent from mesenchyme. During subsequent developmental stages (14.5 dpc), the distribution of MK and HB-GAM became more restricted and the staining was localized only in parts of the olfactory epithelium (Fig. 2A,B). As elsewhere, the levels of the MK protein decreased by 16.5 dpc, whereas a faint HB-GAM staining was detected in epithelium.

Hair and whisker follicles
Intense MK and HB-GAM staining was observed on the surfaces of both epidermal and mesenchymal cells located at the sites of the hair and whisker follicles (Figs 2A,B, 7Da). When the epithelium started to invaginate the underlying condensing mesenchyme, MK staining was present in both components of the developing whisker follicles (Figs 2A, 7Ca), whereas the HB-GAM protein was located only in ectodermal cells (Figs 2B, 4C,D). At this developmental stage (14.5 dpc), nerve endings surrounding the whisker follicles were positive for HB-GAM (Fig. 4E). At 16.5 dpc, a very weak MK reactivity was found only in the mesenchymal component of the whisker follicle. Faint HB-GAM staining was detected in the epithelium and in some mesenchymal cells.

Respiratory system
At 12 dpc, the endoderm-derived tracheal epithelium and the adjacent mesenchyme exhibited strong MK immunoreactivity, whereas the staining with HB-GAM was weak in epithelium and absent from mesenchyme (data not shown). From 13 to 16.5 dpc, MK protein was distributed on the surfaces of epithelial cells of the intrapulmonary segmental bronchi, bronchioli, alveolar ducts, and surrounding lung parenchyma (Figs 2B, 5Fa). The distribution pattern of MK in bronchial epithelium was related to its differentiation status: the protein was expressed in differentiating bronchial epithelial cells, but it diminished and disappeared completely from differentiated cells. In contrast, faint HB-GAM staining was observed in bronchial epithelial cells, whereas the surrounding lung parenchyma was negative (Figs 2B, 4G, 5Fb).

Digestive system
From 12 to 14.5 dpc, MK and HB-GAM proteins were found at sites of the oral epithelium where palatal rugae are formed (Figs 2A,B, 7Fa). The epithelium of the tongue exhibited also strong MK and HB-GAM staining (Figs 2A,B, 3D, 7Fa). In the developing submandibular salivary gland, both proteins were localized on the cell surfaces of the branching epithelial buds, whereas only MK staining was found in basement membranes and in mesenchyme (Figs 3H, 4H, 7Ba). In the esophagus, MK and HB-GAM staining were detected on the surfaces of epithelial cells, whereas the mesenchymal cells and the basement membranes were negative (Fig. 2A,B). In the stomach and intestine, MK protein was found in both mucosal epithelium and mesenchyme (Figs 2A, 3A,G, 5Fa). The distribution of HB-GAM protein was almost similar to that of MK in the developing stomach but the staining was weaker, whereas a moderate staining was observed only in the mucosal epithelium of intestine (Figs 2B, 5Hb). MK and HB-GAM reactivity were also observed in the outer mesenchymal layer of the stomach and the intestine, which gives rise to the smooth muscle layer (Fig. 3G). At 16.5 dpc, the MK staining persisted in the esophagus, stomach, and intestine (data not shown). In the duodenum, the MK immunoreactivity was distributed in both epithelium and mesenchyme, whereas in midgut the staining was found in mesenchymal cells surrounding the mucosa. In contrast, HB-GAM staining was observed only in epithelial cells of intestinal mucosa.

Fig. 6. Localization of syndecan-1 in different tissues of 12.5-13 dpc mouse embryos. The staining is detected in epithelial, mesenchymal, and neuronal cells. (A) Meninges (m) and cranial epithelium (ce). (B) Prevertebrae. (C) Lung. (D) Liver. Bar, 25 µm.
Urogenital system
From 12.5 to 13.5 dpc, both MK and HB-GAM proteins were present in the mesenchyme and tubules of the developing metanephros (Figs 3A, 5Ga,b). From 14.5 to 16.5 dpc, MK was distributed in parenchyma, basement membrane and epithelium of nephrogenic tubules of the kidney, whereas the HB-GAM staining was absent from the mesenchymal cells surrounding the developing metanephros. Both proteins were localized on the surfaces of epithelial cells and in the peripheral and central core mesenchyme of the genital tubercle (data not shown). At 16.5 dpc, only HB-GAM staining persisted in the genital organ.

Facial processes and limb buds
In facial processes and developing limbs (from 10 to 14.5 dpc), strong MK and HB-GAM reactivities were detected in epithelium and underlying proliferating mesenchyme (Figs 2A,B, 3D, 7Aa), whereas the underlying proliferating mesenchyme and the basement membrane exhibited strong staining only for MK (Fig. 3J). At 16.5 dpc, MK was absent from the facial processes (data not shown), whereas in limbs the staining was restricted to the mesenchyme situated at the distal part of the developing digits (Fig. 5Ja). Weak HB-GAM reactivity was observed only in the surface epithelium of the facial processes and digits (Fig. 5Jb).

Fig. 7. Comparison of the distribution of MK (Xa) and syndecan-1 (Xb) in different tissues of 13-14.5 dpc mouse embryos. (A) Limbs at 13 dpc. (B) Submandibular gland at 14 dpc. (C) Developing whisker follicles at 13.5 dpc. (D) Developing hair follicles and blood vessels (arrows) at 14.5 dpc. (E) Dorsal root ganglia (gg) at 13 dpc. (F) Palatal rugae (arrows) and tongue (tg) epithelium from 14.5 dpc embryo. Abbreviation: oe, oral epithelium. Bars, 50 µm.
Immunolocalization of syndecan-1 in mouse embryos

Previous data have demonstrated syndecan-1 expression in embryonic mouse tissues undergoing epithelial-mesenchymal interactions, such as the developing limb buds (Solursh et al., 1990), kidney (Vainio et al., 1989) and whisker follicles (Trautman et al., 1991). We studied the expression of syndecan-1 in 12.5-14.5 dpc mouse embryos. At 12.5 and 13.5 dpc, the patterns of syndecan-1 distribution in developing tissues and organs corresponded largely to those of MK and HB-GAM. The proteoglycan was widely distributed in epithelia of the skin, sensory organs, tongue, esophagus, stomach, gut, pancreas, and ureter (Figs 6C, 7Bb,Cb,Db,Fb). Syndecan-1 staining was found in both epithelium and mesenchyme of several organs undergoing epithelial-mesenchymal interactions, such as whisker follicles, teeth, salivary glands, lung and kidney. The mesenchyme of the central and peripheral nervous systems at this developmental stage. Earlier findings suggest that syndecan-1 is not expressed in adult central nervous system. However, syndecan-1 mRNA transcript of unusual size; (4.5×10^3 M_r, usually 3.4 and 2.6×10^3 M_r) has been found in mouse brain (Saunders et al., 1989). A diffuse staining was observed on cell surfaces of the developing brain (neopallial cortex, midbrain, diencephalon) and neural tube. Intense syndecan-1 staining was also found in the meninges (Fig. 6A). In the peripheral nervous system, the staining was evident in the roots of the ganglia and in neuronal projections, whereas the bodies of the ganglia were negative (Fig. 7Eb).

At 14.5 dpc, syndecan-1 distribution became more restricted and several previously stained organs were negative (Fig. 2C). This distribution corresponded in part with the patterns of MK and HB-GAM protein expression (see table 1). Syndecan-1 reactivity persisted in skin, palate, tongue, esophagus, stomach and gut epithelium, whereas in the olfactory organ epithelium, only the respiratory portion was positive. In the vast majority of organs and tissues undergoing epithelial-mesenchymal interactions (e.g. whisker follicles, teeth) a strong staining was detected in both epithelium and mesenchyme, corresponding with the distribution of MK and HB-GAM. In contrast, syndecan-1 staining was faint and punctuated in lung, heart, and stomach. Furthermore, several tissues expressing MK and HB-GAM reactivity were negative for syndecan-1: the staining was absent from kidney and neuronal structures.

Localization of MK transcripts in mouse embryos

Our in situ hybridization results confirm the previous data of Kadomatsu et al. (1990) on MK mRNA expression in 11 and 13 dpc mouse embryos, and show that in the vast majority of the developing organs the patterns of mRNA expression correlate to those of the MK protein. In 13 to 14.5 dpc mouse embryos, MK transcripts were detected in the mesenchyme of the facial processes (Fig. 8A,B), limbs, genital tubercle and tail (Fig. 8E). In organs undergoing epithelial-mesenchymal interactions such as the olfactory organ (Fig. 8B), inner ear, lung, and gut (Fig. 8F) MK transcripts were expressed in both epithelium and mesenchyme. Neuronal tissues including the brain (Fig. 8A,D) and neural tube expressed MK mRNA, whereas the signal was absent from cell bodies in the ganglia (Fig. 8A).

Fig. 8. MK mRNA expression in various developing tissues and organs in sagittal sections of 13 and 14.5 dpc mouse embryos. (A) Maxillary process, tongue (tg), and brain (b) at 13 dpc. The signal is absent from the trigeminal ganglia (gg). (B) Facial processes (f), olfactory organ (o), and tongue (tg) at 13 dpc. (C) Section of the same area as in B, labeled with a sense probe. (D) Brain (neopallial cortex) of a 14.5 dpc embryo. Note that the choroid plexus (arrow) is labeled. (E) Genital tubercle (gt) and tail (tl) at 14.5 dpc. (F) Intestine (i) and pancreas (pn) at 14.5 dpc. Bar, 200 µm.
The choroid plexus, which was negative for MK protein, intensely expressed MK transcripts (Fig. 8D, arrow). No specific signal was detected with sense probe at any developmental stage throughout the study (Fig. 8C).

At more advanced developmental stages (15 to 19 dpc), Kadomatsu et al. (1990) reported that the MK hybridization signal was detectable only in the kidney. However, slot blot analysis has shown that MK mRNA is also expressed in the head of later-stage embryos (Nakamoto et al., 1992). Our results show that the MK mRNA signal persists, at least until 16.5 dpc, in the brain, facial processes (Fig. 9D), soft palate (Fig. 9E), olfactory organ (Fig. 9F), teeth, limbs, intestine, kidney and genital tubercle (data not shown). Interestingly, we were not able to detect MK protein expression by immunohistochemistry in several tissues expressing the MK transcripts such as in facial processes and soft palate.

**MK and HB-GAM bind to syndecan-1 in a dose-dependent manner**

MK and HB-GAM are growth-associated molecules that bind heparin, and it is thus likely that cell surface HSPGs are involved in the regulation of cellular responses to these proteins. This hypothesis relies on the assumption that cell surface HSPGs are able to recognize MK and HB-GAM. We therefore tested the binding of MK and HB-GAM to syndecan-1 in a solid-phase assay using nitrocellulose-immobilized proteins and metabolically 35SO4-labeled syndecan-1 from mouse mammary epithelial cells. Syndecan-1 bound both MK and HB-GAM in a dose-dependent manner (Fig. 10A). Syndecan-1 bound MK and HB-GAM very similarly as equimolar amounts of type I collagen. The largest amounts (200 pmol) of proteins resulted in about 2- and 5-fold more syndecan-1 being bound to HB-GAM and MK respectively, as compared to type I collagen (Fig. 10A). HS (50 µg/ml) effectively reversed the binding to each protein (Fig. 10B). CS, in turn, had no effect on the binding of syndecan-1 to collagen but inhibited binding to MK and HB-GAM by about 60% (Fig. 10B).

**MK and HB-GAM do not induce syndecan-1 expression or cell proliferation in limb and facial mesenchyme**

Analysis of syndecan expression by whole-mount immunohistochemistry did not reveal induction of this molecule around the beads releasing MK or HB-GAM protein (Fig. 11A). However, the expression of syndecan-1 was induced in the mesenchyme contacting the recombinated epithelium (arrows).

Cell proliferation was analysed by labeling the explants with bromodeoxyuridine (BrdU). In recombinants of epithelium and mesenchyme of the hindlimb or jaw, the epithelium induced cell proliferation (arrows), whereas the beads releasing either MK or HB-GAM were not able to stimulate cell proliferation (Fig. 11B,C).

**DISCUSSION**

**Developmental roles of MK and HB-GAM**

Our immunohistochemical studies demonstrated characteristic distribution patterns for midkine (MK; Kadomatsu et al., 1988) and heparin binding-growth associated molecule (HB-GAM or pleiotrophin; Rauvala, 1989), members of a novel family of heparin-binding molecules (Muramatsu, 1993), during mouse embryonic development (9 to 16.5 dpc). Both proteins are expressed in the central and peripheral nervous systems. Several in vitro experiments have shown that MK and HB-
GAM may initiate and maintain the differentiated state of neuronal cells (Li et al., 1990; Michikawa et al., 1993; Muramatsu and Muramatsu, 1991; Muramatsu et al., 1993; Raulo et al., 1992; Rauvala, 1989; Rauvala et al., 1994).

The appearance of MK and HB-GAM staining in many organs undergoing branching morphogenesis (e.g. salivary gland, lung, kidney) was concomitant with epithelial differentiation, suggesting that these molecules may be involved in the differentiation process of several epithelial cell lineages.

Another interesting aspect of MK and HB-GAM distribution was their specific localization in the basement membranes of developing organs. At 16.5 dpc, weak MK staining decorated the basement membranes of several organs (e.g. salivary gland, lung), which ceased to express MK mRNA, suggesting that the embryonic basement membranes may serve as sites of storage for the protein.

MK and HB-GAM expression was often detected at sites of cartilage and bone formation. The first stage of endochondral bone formation involving the transformation from mesenchyme to cartilage correlated with the expression of MK on the surfaces of the condensed mesenchymal cells. Furthermore, direct conversion of mesenchymal cells into osteoprogenitors (intramembranous ossification) correlated with the expression of both MK and HB-GAM. Hence, these molecules may play a regulatory role during initiation of cartilage and bone development.

Comparison of the expression pattern of MK mRNA and the corresponding protein in developing organs are in line with both autocrine and paracrine modes of action. In some cases the MK immunoreactivity was absent from sites of mRNA expression (e.g. chordoid plexus; see arrows at Figs 3B, 8D), suggesting either paracrine effects or inability to translate it. Our immunohistochemical analysis showed that HB-GAM was distributed principally in basement membranes and epithelial cell surfaces, whereas, in an earlier study, Vanderwinden et al. (1992) showed that the HB-GAM transcripts were mainly localized in neuroectodermal and mesenchymal cell lineages. Hence, it is apparent that the expression of the mRNA does not always correspond to that of the protein, suggesting a paracrine mechanism of action for HB-GAM in several developing organs. In the developing nervous system, however, both HB-GAM transcripts and the protein seem to localize to the same areas, suggesting an autocrine mechanism of action. For more definitive clues on how MK and HB-GAM might act it is essential to indentify their cell surface receptors.

It is not clear at present whether the biochemical modes of action of MK and HB-GAM are similar. Most of the results obtained to date support the notion that MK and HB-GAM function autonomously, but this does not exclude the possibility that they could interact as well. MK and HB-GAM were frequently localized in the same developing organ, often with overlapping patterns of expression. RA induces the expression of MK (Matsubara et al., 1994; Muramatsu, 1993) whereas HB-GAM expression is not affected by RA (Merenmies, 1992). Furthermore, no available information exists about 'cross-talk' between the individual genes (i.e. whether HB-GAM could be induced by MK or vice versa). Analysis in transgenic animals of either loss- (knock out) or gain-of-function (i.e. ectopic expression) mutation, may contribute to our understanding of MK and HB-GAM function in embryonic development.

**Fig. 10.** (A) Binding of syndecan-1 to MK, HB-GAM, and type I collagen (COL I). The proteins were immobilized onto nitrocellulose filters using a vacuum blotting apparatus. The filter was incubated with phosphor imaging equipment. Results represent the mean ± s.e.m. of two independent analyses and are expressed as percentages of binding to type I collagen. Amounts binding to BSA have been subtracted from the values. (B) Effect of the free glycosaminoglycans on the binding of syndecan-1 to MK, HB-GAM, and type I collagen (COL). The binding of 35SO4-labeled syndecan-1 to proteins immobilized onto nitrocellulose filters was assessed in the presence of 50 µg/ml heparan sulfate (HS), chondroitin sulfate (CS), or both (HS + CS). Control represents the binding in the absence of HS or CS. Values are expressed as percentages of uninhibited binding and are obtained using phosphor imaging analysis of filter-bound radioactivity.

**Association of MK and HB-GAM with epithelial-mesenchymal interactions during organ development**

Studies in experimental embryology have demonstrated that organ development depends on sequential and reciprocal interactions between epithelial and mesenchymal tissues (Gumbiner, 1992; Saxén, 1972; Saxén et al., 1980). MK and HB-GAM were expressed in many developing organs, such as...
Midkine and HB-GAM in mouse embryos

49 glands, kidney, lung, gut, whisker follicles and teeth, and their expression correlated with inductive tissue interactions. For instance, in the whisker follicle (sensory hair or vibrissa), MK and HB-GAM were first intensely expressed in the thickened epithelium. When the epithelium started to invade the underlying mesenchyme, cells of the prospective root papilla mesenchyme started to express MK immunoreactivity. Although the mRNA expression for HB-GAM has been reported to occur in the mesenchymal sheet of the whiskers (Vanderwinden et al., 1992), the protein was detected in epithelium, again suggesting paracrine action.

In developing limb buds, both MK and HB-GAM proteins were localized in the epithelium of the apical ectodermal ridge and the mesenchyme of the progress zone. A similar distribution pattern was observed in the facial processes. The development of the limb buds and facial processes is dependent upon interaction between mesenchyme and epithelium (Richman and Tickle, 1989; Tickle, 1991; Wedden, 1987). It has been demonstrated by tissue recombination experiments that the epithelium regulates facial and limb outgrowth by controlling both the maintenance (Richman and Tickle, 1989; Wedden, 1987) and the proliferation (Minkoff, 1991; Niswander et al., 1993; Reiter and Solursh, 1982) of mesenchymal cells at the apex of the facial processes and limb buds. Taken together, these observations suggest that MK and HB-GAM may be involved in signalling during epithelial-mesenchymal interactions.

Syndecan-1 may regulate the biological effects of MK and HB-GAM

Syndecan-1, a cell surface heparan sulfate proteoglycan, is expressed in morphogenetically active mesenchymal cells during the development of facial processes, limb buds, whisker follicles, kidney, and tooth. Its expression has been shown to be regulated by epithelial-mesenchymal interactions in several organs (Bernfield et al., 1992; Thesleff et al., 1991; Vainio et al., 1989) and our present results indicate that this is the case also in facial processes and limb buds. MK showed striking co-localization with syndecan-1 in many mesenchymal cell populations, and HB-GAM was expressed at some, although not all, of these sites (see Table 1). Our studies indicate that MK and HB-GAM bind to syndecan-1, but that they do not induce syndecan expression in facial and limb mesenchyme when released from agarose beads. Syndecan-1 influences the binding and mitogenic activity of FGF-2 and hence it may act as a low affinity growth factor receptor (Jalkanen et al., 1993; Salmivirta et al., 1992). Syndecan-1 can bind simultaneously to FGF-2 and ECM molecules such as fibronectin and type I collagen (Salmivirta et al., 1992), and overexpression of syndecan-1 inhibits the biological effects of both FGF-1 and FGF-2 in 3T3 cells (Mali et al., 1993). Hence, syndecan-1 may regulate the biological functions of FGFs in multiple ways during organogenesis (Ruoslahi and Yamaguchi, 1991), and it is possible that similar regulatory mechanisms exist for MK and HB-GAM.

It is apparent that MK, HB-GAM, FGFs and syndecan-1 are frequently colocalized in embryonic tissues (Gonzalez et al., 1990; Niswander and Martin, 1992; our present results), suggesting molecular interactions. For example, the binding of MK and HB-GAM to syndecan-1 in embryonic mesenchyme could considerably reduce the free heparan sulfate sites of the proteoglycan, thereby affecting the binding and biological activities of FGFs. In our assay system, MK and HB-GAM proteins that were released from the beads in limb and jaw mesenchyme did not stimulate cell proliferation, whereas FGFs are mitogens for these cells. However, expression of MK and HB-GAM in these tissues correlates with active cell division. The effect of MK and HB-GAM on cell proliferation appears to depend on cell type: in several cell lines they do not affect the mitogenic activity, whereas they may be mitogens for some other cell populations (Hampton et al., 1992; Li et al., 1990; Muramatsu and Muramatsu, 1991; Nurcombe et al., 1992; Raulo et al., 1992). Taken together these findings suggest that molecular interactions may exist between FGFs, MK, HB-GAM and syndecan-1 during development, and that these molecules participate in the spatial and temporal control of morphogenesis.
REFERENCES


(Accepted 29 September 1994)