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The large functional spectrum of the heparin-binding cytokines MK and HB-GAM in continuously growing organs: The rodent incisor as a model

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ABSTRACT

The heparin binding molecules MK and HB-GAM are involved in the regulation of growth and differentiation of many tissues and organs. Here we analyzed the expression of MK and HB-GAM in the developing mouse incisors, which are continuously growing organs with a stem cell compartment. Overlapping but distinct expression patterns for MK and HB-GAM were observed during all stages of incisor development (initiation, morphogenesis, cytodifferentiation). Both proteins were detected in the enamel knot, a transient epithelial signaling structure that is important for tooth morphogenesis, and the cervical loop where the stem cell niche is located. The functions of MK and HB-GAM were studied in dental explants and organotypic cultures in vitro. In mesenchymal explants, MK stimulated HB-GAM expression and, vice-versa, HB-GAM upregulated MK expression, thus indicating a regulatory loop between these proteins. BMP and FGF molecules also activated expression of both cytokines in mesenchyme. The proliferative effects of MK and HB-GAM varied according to the mesenchymal or epithelial origin of the tissue. Growth, cytodifferentiation and mineralization were inhibited in incisor germs cultured in the presence of MK neutralizing antibodies. These results demonstrate that MK and HB-GAM are involved in stem cells maintenance, cytodifferentiation and mineralization processes during mouse incisor development.

Introduction

MK (midkine) and HB-GAM (heparin-binding growth associated molecule) constitute a distinct family of heparin-binding growth/differentiation factors (Maruta et al., 1993; Obama et al., 1994). MK is encoded by a retinoic acid (RA) responsive gene (Kadomatsu et al., 1988; Tomomura et al., 1990) and shares approximately 50% sequence homology with HB-GAM (Merenmies and Rauvala, 1990; Rauvala, 1989), which is also called pleiotrophin (PTN) (Li et al., 1990) or heparin-binding neurotrophic factor (HBNF) (Bohlen et al., 1991). Both molecules are produced as precursors, and the mature proteins are formed by cleaving-off residue signal peptides of about 3 kDa. The secreted forms of MK (13 kDa) and HB-GAM (15 kDa) are highly conserved among species (Kurtz et al., 1995; Obama et al., 1994; Tsutsui et al., 1991). MK shows 87% identity between the human and the murine proteins (Tsutsui et al., 1991), while sequence homology among human and murine HB-GAM exceeds 99% (Li et al., 1990). MK and HB-GAM are structurally unrelated to other heparin-binding factors, such as fibroblast growth factors (FGFs), hepatocyte growth factor (HGF) and plateled-derived growth factor (PDGF) (Maruta et al., 1993; Obama et al., 1994). The receptors for MK and HB-GAM have not yet been well characterized. Cross-linking studies have shown that MK and HB-GAM bind to syndecan-1 and -3 respectively (Mitsiadis et al., 1995b; Raulo et al., 1994). Interestingly, MK and HB-GAM bind to nucleolin (Take et al., 1994), a protein located on the cell surface that acts as a shuttle between the cytoplasm and the nucleus, suggesting that another signal transduction mechanism may exist for MK and HB-GAM.

Both proteins stimulate neurite outgrowth (Muramatsu and Muramatsu, 1991; Muramatsu et al., 1993; Rauvala, 1989), angiogenesis (Mashour et al., 2001), enhance bone formation and cartilage differentiation (Dreyfus et al., 1998; Imai et al., 1998; Ohta et al., 1999), but controversy exists regarding their mitogenic effects (Li et al., 1990; Maruta et al., 1993; Mashour et al., 2001; Mitsiadis et al., 1995b; Muramatsu and Muramatsu, 1991; Rauvala, 1989). Their preferential localization in a variety of developing tissues and organs undergoing epithelial-mesenchymal interactions (Mitsiadis et al., 1995b; Vanderwinden et al., 1992) suggests a role for MK and HB-GAM in the regulation of organogenesis.

Teeth are organs whose development depends on sequential and reciprocal interactions between cells of the oral epithelium and cranial neural crest-derived mesenchyme (Cobourne and Mitsiadis, 2006; Lumsden, 1988; Mitsiadis, 2001). Functional differentiation of dental mesenchymal and epithelial cells gives rise to odontoblasts secreting

Abbreviations: HB-GAM, heparin binding-growth associated molecule; MK, midkine.

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the organic matrix of dentin and ameloblasts producing the enamel matrix. The molecular mechanisms involved in tooth formation have started to become well known. Secreted signaling molecules, transcription factors and extracellular matrix molecules are expressed in different stages of mouse tooth development and their deletion may affect odontogenesis (reviewed by Mitsiadis, 2001; Tucker and Sharpe, 2004).

Previous studies have shown that MK is expressed in the developing mouse molar teeth and affects their morphogenesis *in vitro* (Mitsiadis et al., 1995a). However, molars differ from the continuously growing incisors of rodents, which provide an excellent model of tissue organization involving defined regions of cell proliferation (stem cell compartment), differentiation and maturation. Another intriguing aspect of these teeth is that lingual dental epithelial cells do not differentiate into ameloblasts and thus cannot synthesize enamel matrix. In the present study we study the expression, regulation and biological functions of MK and HB-GAM in the different developmental stages of the mouse incisor.

Materials and methods

Animals and tissue preparation

Swiss mice were used at embryonic and postnatal stages. Embryonic age was determined according to the vaginal plug (day 0) and confirmed by morphological criteria. The heads from the embryonic day-10 (E10) to the E18 mouse embryos were dissected in Dulbecco's phosphate-buffered saline (PBS), pH 7.4. Mandibles and lower incisors were removed under a stereomicroscope in PBS, pH 7.4, and fixed in 4% paraformaldeyde (PFA) for 24 h at 4 °C.

Immunohistochemistry and in situ hybridization on tissue sections

Affinity-purified antibodies against mouse MK and rat recombinant HB-GAM proteins were produced in rabbits as previously described (Muramatsu et al., 1993; Rauvala, 1989). These polyclonal antibodies have been characterized by Western blotting and their specificity has been verified by immunohistochemistry (Mitsiadis et al., 1995b). A rat anti-mouse syndecan-1 antibody (281-2) was also used. Preparation and characterization of this monoclonal antibody has been described earlier (Jalkanen et al., 1985).

Immunoperoxidase (ABC kit, Vector Laboratories, Burlingame, CA) and immunofluorescence staining was performed as previously described (Mitsiadis et al., 1992, 1995b). Positive peroxidase staining produces red/brown color on light microscopy. Replacement of primary antibodies with non-specific rabbit IgG served as a negative control.

For *in situ* hybridization, both [^{35}S]UTP- and digoxigenin-labeled sense (pSP64) and antisense (pSP65) mouse *MK* riboprobes were used. *In situ* hybridization on either paraffin sections or cryosections was performed as described earlier (Mitsiadis et al., 1998, 1995b).

Dental tissue explants

The cervical parts of the incisor tooth germs were carefully dissected in Dulbecco's PBS from the rest of the E15 to E16 incisors. The cervical region contains both proliferating epithelial and mesenchymal stem cells. After dissection, tissues were incubated for 3 min in 2.25% trypsin/0.75% pancreatin on ice. Epithelial and mesenchymal tissues were mechanically separated under a stereomicroscope. Explants of either isolated dental epithelia or mesenchyme were cultured on polycarbonate membranes (pore size 0.1 mm; Costar, USA) supported by metal grids (Trowell-type). The explants were cultured for 2 to 20 h in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal calf serum (FCS; Gibco) in a humidified atmosphere of 5% CO_2 in air at 37 °C. After culture, the explants were fixed overnight in 4% PFA, and were treated with antibodies as whole mounts. Some of the explants were used for cell proliferation analysis.

Recombinant proteins and treatment of beads

Recombinant MK (30 μg/ml) and HB-GAM (from 5 to 20 μg/ml) proteins were stored at -70 °C in 0.05 M Na-phosphate containing 1.0 M NaCl until use. Recombinant EGF (10 μg/ml) and FGF10 (100 μg/ml) proteins were from Boehringer Mannheim Corp. (Germany). Recombinant BMP2 and BMP4 proteins (1.12 mg/ml) were from Genetics Institute (Cambridge, Massachusetts, USA). The proteins were stored at −70 °C in 0.5 mM arginine–HCl, 10 mM histidine (pH 5.6) until use.

Affi-gel agarose beads (75–150 μm diameter; BioRad Labs.) were used as carriers of MK, HB-GAM, EGF, FGF10, BMP2 and BMP4 proteins (Mitsiadis et al., 1995a). Anion exchange resin beads (AG 1-x2, 106–205 μm diameter; BioRad Labs.) were used as carriers of retinoic acid (RA). Beads were washed once with PBS and pelleted. Recombinant proteins were diluted into PBS, pH 7.4, to concentrations 50–200 ng/μl/5 μl/50 beads (EGF, 200; MK, HB-GAM and FGF10, 50–150; BMP2 and BMP4, 200) and incubated for 30 min at room temperature. Beads used as carriers for RA were washed for 5 min with dimethyl-sulfoxide (DMSO; Merck). RA was diluted into DMSO to concentrations 100 ng/μl/500 μl/50 beads and incubated for 30 min at RT. Beads were washed for 5–15 min in culture media and then transferred with a mouth-controlled capillary pipette on top of the explants. Control beads for MK were treated identically with 0.1% BSA in PBS, whereas DMSO beads were used as controls for RA.

Whole-mount BrdU labeling and immunohistochemistry of dental explants

Cell proliferation was analyzed by using a cell proliferation kit (Boehringer Mannheim, Germany). After culture, the explants were labeled for 1 h with bromodeoxyuridine (BrdU) according to the manufacturers instructions and as described earlier (Mitsiadis et al., 1995a). They were fixed in 4% PFA overnight at 4 °C, treated to inhibit endogenous peroxidase with 3% H_2O_2/PBS for 30 min at room temperature, washed in PBS, and used immediately for staining. Whole mount immunohistochemistry with antibodies against MK, HB-GAM and BrdU was performed as earlier described (Mitsiadis et al., 1995a). When the color reaction was satisfactory, the explants were washed in tap water and mounted in Aquamount (Gurr, England).

Organotypic cultures

Incisor tooth germs were carefully dissected from the mandibles of E16 mouse embryos in PBS. The incisors were cultured in Trowell-type cultures, and the basic culture medium (DMEM and 10% FCS) was supplemented with neutralizing antibodies against the MK protein (40 μg/ml). The neutralizing ability of these antibodies has been already demonstrated either in cell cultures (neuronal and Wilm's tumour cells) (Muramatsu et al., 1993) or in organotypic cultures (molar tooth germs) (Mitsiadis et al., 1995b). In control cultures, normal rabbit serum (NRS) was added to the medium in an amount identical to the anti-MK antibody. The incisor germs were cultured for 6 to 7 days with three changes of fresh medium. After culture, the incisors were fixed in 4% PFA overnight at 4 °C, rinsed with PBS, dehydrated in ethanol and embedded in paraffin. Immunohistochemistry was performed in 5 μm serial sections by omitting the primary antibody (anti-MK) and thereafter the sections were counterstained with hematoxylin. Sections of incisors cultured in presence of neutralizing antibodies were slightly counterstained to better reveal the MK staining.

Results

MK gene expression in the developing incisor

In situ hybridization analysis showed a ubiquitous expression of *MK* transcripts in both dental and non-dental tissues of E10–E13 mouse embryos (data not shown; Mitsiadis et al., 1995a). From the cap stage (E14–E15), *MK* gene expression became progressively restricted to the incisor tooth germ and at E16.5–E17, *MK* was strongly expressed in dental papilla mesenchyme and cells that differentiate into odontoblasts (Figs. 1A and D) while in the epithelium a very strong hybridization signal for *MK* was detected in the cervical loop area where the stem cell niche is located (Fig. 1A). Expression was also observed in other parts of the dental epithelium and in osteogenic areas of the mandible (Figs. 1A and D). No specific hybridization signal was detected with the MK sense probe (Fig. 1B). At E18, the strong hybridization signal persisted in odontoblasts and dental papilla cells, whereas a moderate signal was detected in dental epithelium (inner enamel epithelium and preameloblasts) (Figs. 1C, E and F).

Distribution of MK and HB-GAM proteins in the developing incisors

At E11, both MK and HB-GAM immunoreactivities were detected in the anterior part of the thickened epithelium of the incisors (Figs. 2A and B). The staining was found on cell surfaces and in the basement membrane underlying the stained epithelial cells (arrows in Figs. 2A and B). A weaker staining for the MK was also detected in the whole basement membrane (arrowheads in Fig. 2B) and in the mesenchyme (Fig. 2B). A strong HB-GAM staining was observed in neuronal structures (asterisk in Fig. 2A). At E13, the epithelium of the incisor forms a bud, around which the mesenchyme condenses. Immunostaining for both proteins was mainly detected on the surfaces of condensing mesenchymal cells that are in close contact