A New Family of Heparin Binding Growth/Differentiation Factors: Differential Expression of the Midkine (MK) and HB-GAM Genes during Mouse Development¹

Masaru Nakamoto, Shyuichiro Matsubara, Teruo Miyauchi, Hiroya Obama, Masayuki Ozawa, and Takashi Muramatsu

Department of Biochemistry, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima, Kagoshima 890

Received for publication, April 20, 1992

MK (midkine) and HB-GAM (heparin-binding growth-associated molecule) constitute a new family of heparin-binding growth differentiation factors. The modes of expression of MK and HB-GAM during mouse development were quantitatively examined by mRNA hybridization. The following three distinct patterns of expression were observed in the brain/head region. On the 11th–13th days of gestation, MK was intensely, but HB-GAM relatively weakly expressed; on the 15th–19th days, both MK and HB-GAM expression became weaker; and in the neonatal period, HB-GAM was intensely expressed and MK expression increased slightly. The level of HB-GAM expression was lower than that of MK in the whole embryo on the 11th to 13th days of gestation. HB-GAM mRNA was detected in the kidney of newborn and young mice, where MK was more highly expressed. The identity of the weakly expressed MK and HB-GAM signals was confirmed by means of the polymerase chain reaction in the neonatal brain (MK), the head of 13-day embryos (HB-GAM), and the kidney of 7-day-old mice (HB-GAM). In conclusion, MK and HB-GAM are frequently co-expressed in the same cells and anatomic regions of the fetus or new born mouse, while their modes of expression differ.

Cytokines or growth factors are important in the regulation of proliferation, differentiation and other cellular activities (1–4). Pleiotropy and redundancy are characteristic features of most cytokines. For example, each interleukin has multiple functions, whereas some of them induce the same cell response (5). Furthermore, members of the FGF family promote the growth of various cells, as well as angiogenesis and mesoderm induction. Acidic FGF and basic FGF display similar functions by binding to their receptors (6, 7).

Among the heparin-binding growth factors, fibroblast growth factors (FGFs) are the best characterized (6). Recently, a new family of heparin-binding growth differentiation factors was identified, which are structurally unrelated to FGF. The initial member of the new family is midkine (MK), which is the product of a retinoic acid responsive gene (8, 9). MK is a secreted protein rich in cysteine and basic amino acids with a molecular mass of around 13,000 (10). The heparin-binding growth-associated molecule (HB-GAM) exhibits about 50% homology with MK at the amino acid level, and all cysteine residues are conserved in MK and HB-GAM (11). HB-GAM has been identified in 4 different laboratories, and has also been called pleiotrophin (12), OSF-1 (13), and HBNF (14). RI-HB, recently identified in chick embryos, which exhibits

65% homology with MK, is probably the chick counterpart of MK (15).

The genomic structures of human and mouse MK are highly conserved in their exons and their exon-intron boundaries and in discrete segments in the 5' upstream regions (16, 17). Moreover, comparison of the murine genomic structures of MK (16) and HB-GAM (18) revealed that these genes have the same exon-intron arrangement, similarly or identically sized exons and highly homologous sequences in exons. These findings strongly suggest that the MK and HB-GAM genes are derived from a common ancestral gene (18).

MK, HB-GAM and RI-HB exhibit neurite extension activity, and some of them also exhibit weak mitogenic activity toward fibroblasts and PC12 pheochromocytoma cells (10–12, 15, 19). In addition, HB-GAM exhibits mitogenic activity toward capillary endothelial cells (20), and is secreted from breast cancer cells and functions as a tumor growth factor (21).

MK is predominantly expressed in the mid-gestation embryo in the mouse (22). *In situ* hybridization revealed that the principal sites of MK expression in mid-gestation mouse embryos were epithelial cells, the brain, mesoderm cells, and the kidney (22). However, *in situ* hybridization experiments lack quantitative information. Furthermore, because of the 52% homology between MK and HB-GAM cDNAs, cross reaction should be rigorously excluded. HB-GAM is principally expressed in neonatal and perinatal brain of the rat (11, 12).

In order to understand the functions of these proteins in mouse embryogenesis, we need precise knowledge on the

¹ This work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

Abbreviations: FGF, fibroblast growth factor; HB-GAM, heparin-binding growth-associated molecule; HBNF, heparin-binding neurotrophic factor; MK, midkine; PCR, polymerase chain reaction; RI-HB, retinoic acid-induced heparin-binding protein.

modes of expression of these proteins. Here, we used probes specific for MK and HB-GAM, and quantitatively analyzed the expression of each heparin-binding protein during mouse development. Because of the neurite extension activity of MK and HB-GAM, we focussed upon the expression of these proteins in the brain. Furthermore, the identity of weak signals was confirmed by means of the polymerase chain reaction.

MATERIALS AND METHODS

Embryos and Organs—Embryos were obtained from ICR mice after natural mating. The day when a vaginal plug was detected was taken as day 0 of pregnancy.

Organs were obtained from day 11–19 embryos and from mice at later stages. The excised organs were immediately frozen in dry ice-ethanol and then stored at -80°C until RNA extraction.

Polymerase Chain Reaction, DNA Sequence Analysis, and Southern Blotting—The polymerase chain reaction (PCR) was performed as described (23) using primers synthesized with a DNA synthesizer (Pharmacia LKB Biotechnology) and templates synthesized from appropriate mRNA with reverse transcriptase. Denaturation was performed at 93°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 3 min. Twenty-five cycles were performed. The PCR products were separated by 1% agarose gel electrophoresis, transferred to nitrocellulose filters, and then hybridized with specific probes (24). In one case, the synthesized DNA recovered from agarose gel electrophoresis was cloned into PUC18 and sequenced by the dideoxy chain termination method (25, 26).

Probes—The MK probe was derived from the 3' untranslated region of mouse MK cDNA [nucleotide numbers 421–674 (9)].

We synthesized a 411 bp fragment by PCR, which corresponds to the 3' untranslated region of the mouse HB-GAM cDNA [nucleotide numbers 722–1132 (13)], using two nucleotide fragments (sense and antisense strands comprising nucleotide numbers 722–731 and 1123–1132, respectively) as primers and cDNA from the brain of 14-day-old mice as a template. The sequence of the PCR product differed by one nucleotide from that published (13), possibly because of a mutation during PCR (nucleotide number 722, A→G). There is no significant sequence homology between these two probes, according to the results of computer analysis. That these MK and HB-GAM probes specifically detect MK and HB-GAM mRNA was confirmed by Northern blotting using mRNA from the head of a 13 day embryo and the brain of a 1-day-old mouse.

Mouse β -actin cDNA used as a reference probe was a gift from Prof. K. Shimada, Osaka University.

RNA Extraction—Total RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction (27). Briefly, organs or cells were homogenized with a Polytron in solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), and then debris was removed by centrifugation at $10,000\times g$ for 15 min. To the supernatant, 0.1 volume of 2 M sodium acetate (pH 4.0), 1 volume of phenol, and 0.2 volume of chloroform were added sequentially. After centrifugation, the aqueous phase was mixed with 1

volume of isopropanol, followed by standing at -20°C for 1 h to precipitate RNA. RNA was pelleted by centrifugation, dissolved in solution D, and then precipitated with isopropanol. After washing with 70% ethanol, the RNA was stored in ethanol at -80°C until RNA analysis.

Poly A⁺ RNA was purified by oligo(dT)-cellulose chromatography from total RNA prepared by the guanidinium, cesium chloride method (24).

RNA Analysis—For slot blot hybridization, 20 μg of total RNA was applied to nitrocellulose filters using an apparatus from Schleicher and Schuell. The filters were hybridized with [^{32}P]dCTP (Du Pont/NEN, 111 TBq/mmol)-labeled cDNA generated by the random oligonucleotide primer method (28) at 42°C overnight, and then washed twice for 10 min with $2\times\text{SSC}/0.1\%$ SDS at room temperature and twice for 30 min with $0.1\times\text{SSC}/0.1\%$ SDS at 55°C .

For Northern blotting, 10 μg of poly A⁺ RNA was denatured with glyoxal, electrophoresed on a 1.0% agarose gel (24), and then blotted onto nitrocellulose filters. The conditions for hybridization and washing of the filters were identical to those used for slot blot analysis.

RESULTS

MK and HB-GAM Expression during Embryogenesis—The expression of MK and HB-GAM messages was examined using RNAs extracted from the brain or head by slot blot analysis (Figs. 1 and 2).

As expected from the previous work (22), MK RNA was highly expressed in the head of day 11 and day 13 embryos. *In situ* hybridization revealed that the sites of the message were the neuroectoderm around the lens and the brain ventricle, etc. (22). MK expression decreased during embryogenesis, but MK RNA was detectable at some level in the head of late embryos and the brain of post-natal mice (Figs. 1 and 2). The slight increase in MK mRNA in the neonatal period is interesting. Previous studies involving *in situ* hybridization showed that MK RNA was not significantly detectable in these regions after day 15 (22). Thus, a more sensitive method allowed the detection of weak MK signals. In this context we also noticed that slot blot analysis showed a very faint signal of MK in the brains of adult mice as well (8).

Since the MK message was weak in later embryos, and in the neonatal and adult brain, it was necessary to confirm its identity in representative cases. We therefore amplified putative MK cDNA from the brain of 1-day-old and adult mice by PCR using specific MK primers. In the neonatal brain, the expected PCR product was present and it hybridized with a specific MK probe (Fig. 3, lane 3). In the adult brain, PCR yielded the product of expected size (data not shown) and the identity of the MK signal was further confirmed by sequence analysis: in nucleotide numbers 195–238, there was no sequence difference from the published one (9). In this particular experiment different primers (a sense strand comprising nucleotide numbers 175–194, and an antisense strand comprising nucleotide numbers 239–258) were used.

HB-GAM was expressed in the head of 11–13 day embryos relatively weakly (Fig. 1). HB-GAM expression decreased as embryogenesis proceeded. In the neonatal brain, HB-GAM expression became intense and the high

Fig. 1. The expression of the MK and HB-GAM genes during mouse development. Total RNAs (20 μ g) from whole embryo (W), head (H), brain (B), liver (L), and kidney (K) were blotted onto nitrocellulose membranes and then hybridized with [32 P]-dCTP-labeled MK, HB-GAM, or β -actin probe. E denotes days of gestation, while P denotes days after birth.

Fig. 2. Quantitative changes in MK (○, □) and HB-GAM (●, ■) expression during head (○, ●) and brain (□, ■) development. The hybridization signals in Fig. 1, quantitated with a LKB Ultrascan XL laser densitometer, were normalized as to the signals of β -actin mRNA and then plotted.

level continued until the 21st day after birth (Figs. 1 and 2). The expression pattern in the neonatal brain is consistent with that in the rat brain (11). The identity of the HB-GAM message in the head of 13 day embryos was also confirmed by PCR (Fig. 3, lane 1). Thus, the postnatal period is the stage when HB-GAM is intensely expressed, whereas the significant level of HB-GAM expression in the mid-gestation period should be kept in mind.

In the present study, we analyzed the head region instead of the brain during embryonic periods, since rapid processing is essential to comparatively measure the mRNA content accurately. However, in the 17 day embryo, we also prepared mRNA from the brain, and measured MK and

Fig. 3. PCR detection of MK and HB-GAM cDNAs. Poly A⁺ RNAs were prepared from 13 day embryo head (lane 1), neonatal kidney (lane 2), and neonatal brain (lane 3). cDNAs synthesized from 1 μ g of poly A⁺ RNAs were used as templates for PCR. Two synthetic oligonucleotides were used as primers; for MK, a sense strand comprising nucleotide numbers 12-31 and an antisense strand comprising nucleotide numbers 464-483 (9); for HB-GAM, a sense strand comprising nucleotide numbers 1-20 and an antisense strand comprising nucleotide numbers

1123-1132 (13). The PCR product was electrophoresed on a 1% agarose gel, transferred to a nitrocellulose filter, and then hybridized with either the [32 P]dCTP-labeled HB-GAM (lanes 1 and 2) or MK (lane 3) probe.

Fig. 4. Comparison of MK and HB-GAM expression between the embryonic brain and the embryonic head region. Total RNAs (20 μ g) from the brain (B) and the head (H) of a day 17 embryo were used for slot blot analysis, as in Fig. 1.

HB-GAM mRNA, and confirmed that they were also low in the brain in that period, although the HB-GAM mRNA level was slightly higher in the brain than in the head (Fig. 4). The strong expression of MK in the head of 11-13 day embryos is principally due to the brain, as shown by *in situ* hybridization (22).

The modes of MK and HB-GAM expression in whole embryos paralleled those in the head. However, the expression in the liver was weak or barely detectable (Fig. 1).

MK and HB-GAM Expression in Neonatal and Perinatal Kidney—MK RNA was detected by *in situ* hybridization at the onset of kidney formation, and was expressed throughout development (22). In adult mice, only the kidney (8) and, to a lesser degree, the uterus expressed MK RNA at significant levels (29). However, no data are available regarding quantitative changes in MK expression during kidney development. We therefore examined this by slot blot analysis. MK RNA was expressed in the kidneys of day 15 embryos, and the expression level remained constant during embryogenesis (data not shown). After birth, MK was expressed most intensely in the neonatal kidney (Fig. 1). In postnatal mice, MK expression in the kidney was distinctly greater than that in other organs. HB-GAM was also expressed in the kidney during development, although at lower levels than that in the brain. That the low level of the HB-GAM mRNA indeed specifies for HB-GAM sequence was confirmed by PCR (Fig. 3, lane 2).

DISCUSSION

The expression patterns of the two members of a new-

heparin binding growth/differentiation factor family, MK and HB-GAM, were compared during mouse development. In the brain/head region, MK was intensely expressed during midgestation (days 11-13 of embryogenesis). Low but significant HB-GAM expression accompanied MK expression in that period. Then, expression of both MK and HB-GAM decreased. HB-GAM expression strongly increased after birth and remained at a high level until 21 days after birth. A slight but significant increase was noted in MK expression during the postnatal period. This period correlates with the rapid growth of the brain, when axons and dendrites grow out, and glial growth starts (30). It should be borne in mind that both MK and HB-GAM enhance neurite outgrowth (11, 12, 19), and it is likely that HB-GAM and, to a lesser extent, MK promote neurite outgrowth *in vivo* at this stage.

MK was intensely and continuously expressed in the developing kidney after birth. HB-GAM was expressed in the kidney at a much lower level. The functions of both factors in nephrogenesis or in the adult kidney remain to be elucidated.

Retinoic acid, which is a derivative of vitamin A, is involved in diverse processes of development, and controls cell differentiation and morphogenesis (31, 32). The expression of MK is induced by retinoic acid, yet there have been no reports indicating the effect of retinoic acid on the expression of HB-GAM. It is possible that HB-GAM expression is controlled by a substance other than retinoic acid, and that such a difference contributes to the different modes of expression of the two heparin-binding growth differentiation factors. Alternatively, HB-GAM expression might also be controlled by retinoic acid, while other requirements necessary for the induction of expression differ between MK and HB-GAM.

The expression of MK and that of HB-GAM in the head of the mid-gestation embryo, the postnatal brain, and the postnatal kidney exhibit a reciprocal relationship. It is important to note that in these regions both MK and HB-GAM messages were detected even though one was weak, and that the identity of the weak message was confirmed by PCR. The biological meaning of the redundant messages and their reciprocal modes of expression are challenging problems to be solved.

We wish to thank Miss Kumiko Sato for her expert secretarial assistance.

REFERENCES

- McMahon, A.P. & Bradley, A. (1990) *Cell* **62**, 1073-1085
- Rosa, F., Roberts, A.B., Danielpour, D., Dart, L.L., Sporn, M.B., & Dawid, I.B. (1988) *Science* **239**, 783-785
- Slack, J.M.W., Darlington, B.G., Heath, J.K., & Godsave, S.F. (1987) *Nature* **326**, 197-200
- Thomas, K.R. & Capecchi, M.R. (1990) *Nature* **346**, 847-850
- Paul, W.E. (1989) *Cell* **57**, 521-524
- Burgess, W.H. & Maciag, T. (1989) *Annu. Rev. Biochem.* **58**, 575-606
- Dionne, C.A., Crumley, G., Bellot, F., Kaplow, J.M., Searfoss, G., Ruta, M., Burgess, W.H., Jaye, M., & Schlessinger, J. (1990) *EMBO J.* **9**, 2685-2692
- Kadomatsu, K., Tomomura, M., & Muramatsu, T. (1988) *Biochem. Biophys. Res. Commun.* **151**, 1312-1318
- Tomomura, M., Kadomatsu, K., Matsubara, S., & Muramatsu, T. (1990) *J. Biol. Chem.* **18**, 10765-10770
- Tomomura, M., Kadomatsu, K., Nakamoto, M., Muramatsu, H., Kondoh, H., Imagawa, K., & Muramatsu, T. (1990) *Biochem. Biophys. Res. Commun.* **171**, 603-609
- Merenmies, J. & Rauvala, H. (1990) *J. Biol. Chem.* **265**, 16721-16724
- Li, Y.-S., Milner, P.G., Chauhan, A.K., Watson, M.A., Hoffman, R.M., Kodner, C.M., Milbrandt, J., & Deuel, T.F. (1990) *Science* **250**, 1690-1694
- Tezuka, K., Takeshita, S., Hakeda, Y., Kumegawa, M., Kikuno, R., & Hashimoto-Gotoh, T. (1990) *Biochem. Biophys. Res. Commun.* **173**, 246-251
- Koveadi, I., Fairhurst, J.L., Kretschmer, P.J., & Böhlen, P. (1990) *Biochem. Biophys. Res. Commun.* **172**, 850-854
- Urios, P., Duprez, D., Le Caer, J.-P., Courtois, Y., Vigny, M., & Laurent, M. (1991) *Biochem. Biophys. Res. Commun.* **175**, 617-624
- Matsubara, M., Tomomura, M., Kadomatsu, K., & Muramatsu, T. (1990) *J. Biol. Chem.* **265**, 9441-9443
- Uehara, K., Matsubara, S., Kadomatsu, K., Tsutsui, J., & Muramatsu, T. (1992) *J. Biochem.* **111**, 563-567
- Naito, A., Yoshikura, H., & Iwamoto, A. (1992) *Biochem. Biophys. Res. Commun.* **183**, 701-707
- Muramatsu, H. & Muramatsu, T. (1991) *Biochem. Biophys. Res. Commun.* **177**, 652-658
- Courty, J., Dauchel, M.C., Caruelle, D., Perderiset, M., & Barritault, D. (1991) *Biochem. Biophys. Res. Commun.* **180**, 145-151
- Wellstein, A., Fang, W., Khatri, A., Lu, Y., Swain, S.S., Dickson, R.B., Sasse, J., Riegel, A.T., & Lippman, M.E. (1992) *J. Biol. Chem.* **267**, 2582-2587
- Kadomatsu, K., Huang, R.-P., Suganuma, T., Murata, F., & Muramatsu, T. (1990) *J. Cell. Biol.* **110**, 607-616
- Kawasaki, E. (1990) in *PCR Protocols-A Guide to Methods and Applications* (Innis, M.A., Gelfand, D.H., Sninsky, J.J., & White, T.J., eds.) pp. 21-27, Academic Press, New York
- Maniatis, T., Fritsch, E.F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York
- Hattori, M. & Sakaki, Y. (1986) *Anal. Biochem.* **152**, 232-238
- Sanger, F., Nicklen, S., & Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
- Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156-159
- Feinberg, A.P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6-13
- Huang, R.-P., Muramatsu, H., & Muramatsu, T. (1990) *Dev. Growth Differ.* **32**, 189-195
- Jacobson, M. (1978) *Developmental Neurobiology*, Plenum, New York
- Roberts, A. & Sporn, M.B. (1984) *The Retinoids* Vol. 2, pp. 209-286, Academic, Orlando, FL
- Thaller, C. & Eichele, G. (1987) *Nature* **327**, 625-628