Regulation and localization of midkine in rat ovary

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Received 14 February 1995

Abstract In a previous experiment, it was shown that midkine (MK) was quite abundant in the follicular fluid; the concentration of MK in bovine follicular fluid was estimated to be 125 μg/l. To investigate the regulation of MK production in the ovary, we examined the effect of pregnant mare's serum gonadotropin (PMSG) and PMSG-hCG treatment on the expression of MK in rat ovary. The mRNA of midkine (MK) was increased by PMSG injection and decreased by PMSG and hCG injection; the profile of change of mRNA of MK was similar to that of FSH receptor. Using in situ hybridization, we observed that the MK mRNA localized to granulosa cells. These results suggest that the granulosa cells produce MK under the control of gonadotropin.

Key words: Midkine; Granulosa cell

1. Introduction

Midkine (MK) is the product of a retinoic acid responsive gene, MK, and constitutes a new family of heparin-binding growth/differentiation factors together with heparin-binding growth-associated molecule (HB-GAM)/pleiotrophin (PTN) [1–7]. MK is transiently expressed in the early stages of embryonal carcinoma cell differentiation and in the mid-gestation period of mouse embryogenesis [1,8,9]. In adult mouse it is significantly expressed only in the kidney and uterus [8,9], but it is widely expressed in adult rat and human tissues [10,11]. It has been reported that MK promotes neurite extension [10,12–14], enhances neuronal survival [4,15–17], and is related to human carcinoma [11,18]. Recently MK was isolated from bovine follicular fluid by using an assay which detects the growth promoting activity on bovine aortic smooth muscle cells. The concentration of MK in bovine follicular fluid was estimated to be 125 ng/ml and this concentration was comparable to those of activin and inhibin [19]. These findings suggest that MK may play a role in the maturation of ovarian follicles. Since the gonadotropic hormones have important regulatory roles in folliculogenesis, we have examined expression of MK mRNA in the ovary of immature rats treated with gonadotropines using both quantitative assays to measure MK mRNA abundance as well as in situ hybridization to examine the cellular distribution of this transcript.

2. Materials and methods

2.1. Animals

Immature female rats of the Wistar strain were primed with 30 IU of pregnant mare's serum gonadotropin (PMSG) alone or 30 IU PMSG and 20 IU of hCG 60 h later. Animals were sacrificed at selected intervals following hormone treatment. The rats were killed and their ovaries were removed, frozen immediately in liquid nitrogen, and stored until use at −80°C .

2.2. Synthesis of the RNA probe

Human MK cDNA was subcloned into the *Bam*HI and *Eco*RI site of the Bluescript KS(+) vector. Digoxigenin-labeled sense or antisense riboprobe RNA were produced from *Eco*RI- or *Bam*HI-digested plasmid DNA by in vitro transcription with T7 or T3 RNA polymerase, respectively, with an RNA labeling kit (Boeringer-Mannheim).

2.3. Extraction of RNA and Northern blot analysis

Total RNA was extracted from the ovaries by the acid guanidinium thiocyanate/phenol/chloroform extraction method. The final RNA pellet was dissolved in diethyl pyrocarbonate treated H_2O. Total RNA was quantified by measuring the absorbance of samples at 260 nm. For Northern blot analysis, 10 μg total RNA was separated by electrophoresis on 1% agarose gels and subsequently transferred to a nylon membrane (Biodyne; ICN) [20,21]. Northern blots were hybridized at 68°C with digoxigenin-labeled antisense riboprobe RNA. Following the standard protocol for the nucleic acid detection kit (Boehringer-Mannheim), membranes were then exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY). The relative abundance of a 1000 base signal in different preparations was analyzed with an LKB 2202 UnitroScan Laser Densitometer (LKB Produker AB, Bromma, Sweden) and standardized against the corresponding amount of β-actin mRNA.

2.4. In situ hybridization

Rat ovaries were embedded into tissue compound and frozen in liquid nitrogen. 7 μm thick sections were cut by a cryostat and mounted on silane-coated glass slides for in situ hybridization. The tissue sections were fixed in 4% paraformaldehyde in PBS and then hybridized with digoxigenin-labeled riboprobe in the hybridization buffer (1 mg/ml yeast tRNA, 20 mM Tris-HCl, pH 8.0, 2.5 mM EDTA, pH 8.0, 1 × Denhardt's solution, 0.3 M NaCl, 50% deionized formamide, 10% dextran sulfate) for 16 h at 50°C. Hybridized sections were then washed with 2 × SSC/50% formamide and 1 × SSC/50% formamide after treatment with RNase A (20 μg/ml in 10 mM Tris-HCl, pH 7.6, 500 mM NaCl, 1 mM EDTA) for 30 min at 37°C. The hybridization material was reacted with alkaline phosphatase-conjugated anti-digoxigenin antibody and then detected by color reaction in the incubation medium containing Nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl-phosphate and levamisole, an inhibitor of endogenous alkaline phosphatase, according to the manufacturer's manual (Digoxigenin Detection kit; Boeringer-Mannheim) [22].

3. Results and discussion

One extremely informative model for studying follicular development has been the hormonally primed immature animal, in which small follicles can be induced to develop in response

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SSDI 0014-5793(95)00231-6

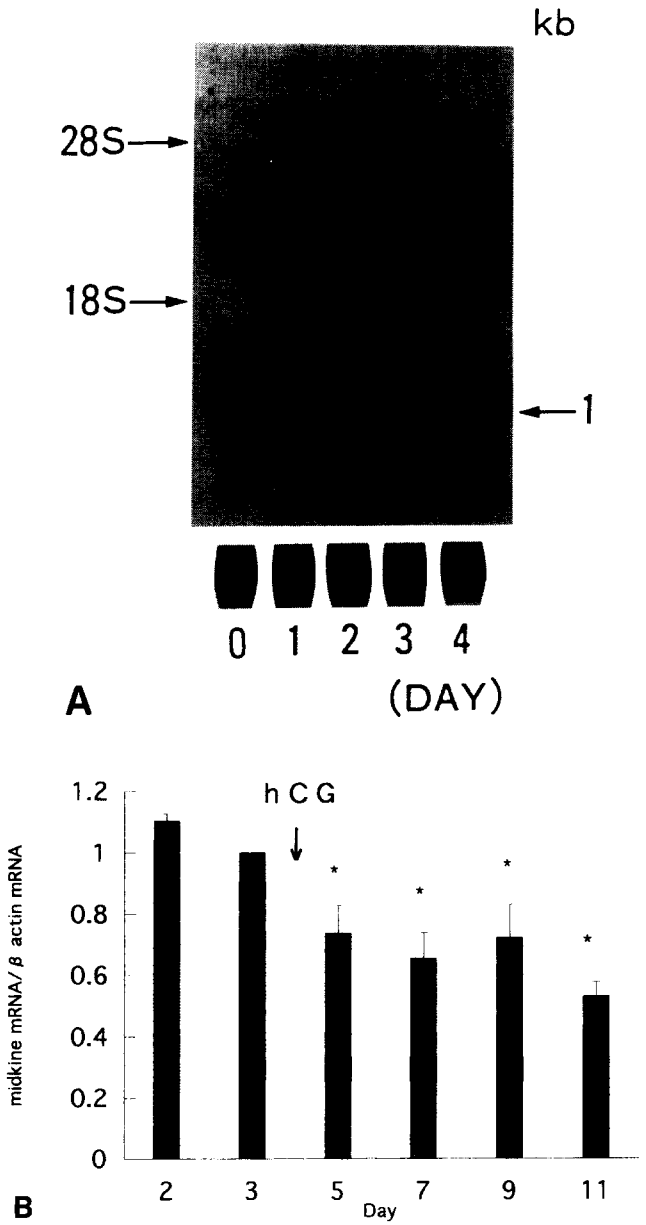

Fig. 1. The effect of PMSG treatment on midkine and β-actin mRNA levels in immature female rat ovary. From each time point 10 μg of total RNA was prepared and fractionated through a 1% agarose gel and blotted as described in section 2. (A) Blots were probed with digoxigenin-labeled midkine cRNA (above) or β-actin cRNA (below). Membranes were exposed to Kodak X-Omat film. (B) Autoradiographs were quantitated by densitometric scanning and the change of midkine mRNA (1000 band) relative to β-actin is expressed as arbitrary units. The mRNA level at each time point is expressed as the fold increase over day 0. The results represent the mean ± S.E.M. of three experiments. *Different from the value of day 0 at $P<0.01$.

to PMSG and to luteinize in response to an ovulatory dose of hCG. Therefore, to determine whether the gonadotropins could modulate levels of MK mRNA, we treated immature rats with PMSG to stimulate follicular development or with PMSG followed by an ovulatory dose of hCG. As shown in Fig. 1, Northern blot analysis of MK revealed a major RNA of 1000 nucleotides, and this transcript was present in the control ovary, reached a peak on day 2, and slightly decreased at day 3. In the previous experiments, the PMSG treatment increased mRNA levels of both FSH and LH/hCG receptor followed by an increase of receptor numbers in the membrane. The mRNA level of FSH receptor increased earlier than that of LH/hCG receptor and slightly decreased by day 4 of the experiment [23]. Taken together, the transcript of MK was induced by PMSG treatment which increased the FSH receptor and stimulated follicular growth. In the same experiment, the PMSG-hCG treatment caused a decrease in FSH receptor mRNA levels and suppressed the LH/hCG receptor mRNA 12–24h after injection; the levels increased to control levels by 48 h and this was followed by a progressive increase [23,24]. Therefore, we tried to investigate the changes of MK mRNA following the administration of hCG to PMSG-primed immature rats. As shown

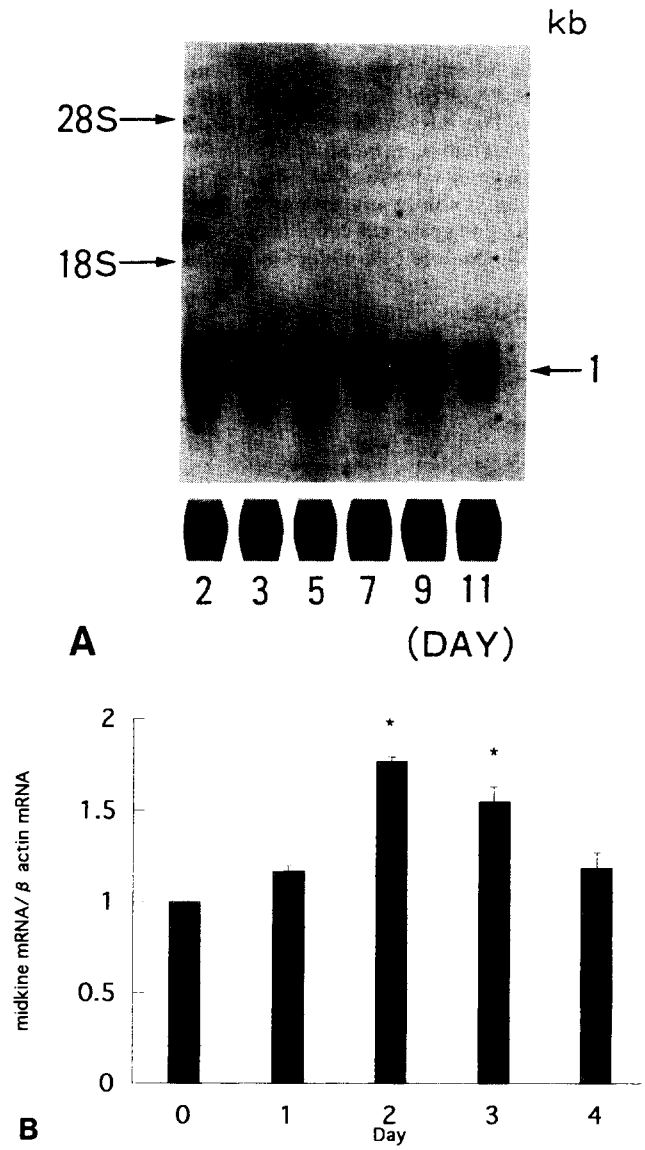

Fig. 2. The effect of PMSG and hCG treatment on midkine and β-actin mRNA levels in immature rat ovary. (A) Midkine mRNA (above), β-actin mRNA (below). (B) Autoradiographs were quantitated by densitometric scanning and the change in midkine mRNA (1000 band) relative to β-actin is expressed as arbitrary units. The mRNA level at each time point is expressed as the fold increase over day 3. The results represent the mean ± S.E.M. of three experiments. *Different from the value of day 3 at $P<0.01$.

Fig. 3. In situ hybridization of the ovary 2 days after hCG treatment with a digoxigenin-labeled riboprobe for midkine. The tissue sections were fixed with 4% paraformaldehyde in PBS and then hybridized with digoxigenin-labeled antisense (A,B) and sense (C) RNA probes in the hybridization buffer. Hybridized sections were then washed as described in section 2. A specific hybridization signal is seen in the granulosa cells of the follicles (A), especially in the cytoplasm of granulosa cells (B), while it is absent in C.

in Fig. 2, Northern blot analysis revealed that hCG injection decreased mRNA of MK. These data showed that the mRNA level of MK was suppressed by hCG injection which stimulated ovulation and luteinization of the ovary. Using both systems, we found that PMSG increased MK mRNA levels and that subsequent administration of hCG to PMSG-primed animals caused a decline in MK mRNA levels to basal values. Although the profile of change of MK mRNA was the same as that of FSH receptor and LH receptor mRNA before hCG injection, after the hCG injection the profile was rather similar to that of FSH receptor mRNA.

Fig. 3 shows in situ hybridizations performed using adjacent ovarian sections from the hormonally treated rats. The MK mRNA was located exclusively to the granulosa cells of healthy follicles. No specific hybridization was observed when sense strand MK RNA probes were used. The time–course of expression of MK responding to the gonadotropins and localization of MK are comparable to those of FSH receptor, which is expressed in mature follicles. Moreover, this suggests that some common regulatory mechanisms for FSH receptor and MK mRNA might be operative in the case of these genes. On the other hand, these results show that the granulosa cells produce MK at least partially under the control of gonadotropin. MK are known to constitute a new family of heparin-binding growth/differentiation factors, which is distinct from the fibroblast growth factor (FGF) family. The present findings show that MK is expressed in granulosa cells and its production may be regulated by gonadotropins: these results do not conflict with the presence of high concentrations of MK in follicular fluid. In a previous study, it was shown that MK was quite abundant in the follicular fluid; the concentration of MK in bovine follicular fluid was estimated to be 125 μg/l. More than 50 ng/ml of MK exerted a growth promoting activity on bovine aortic smooth muscle cells [19]. Although the previous studies suggested that MK might play a role in fetal development, bone formation and brain function [6,14,25], the precise function of this factor remains unknown. To elucidate whether MK exert the growth promoting activity on the granulosa cells will require further experiments.

Acknowledgments: This study was supported by a grant from the Ministry of Education, Science and Culture of Japan (05671353).

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