Effect of retinoic acid on midkine gene expression in rat anterior pituitary cells

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Abstract. Retinoic acid (RA) is converted from retinal by retinaldehyde dehydrogenases (RALDHs) and is an essential signaling molecule in embryonic and adult tissue. We previously reported that RALDH1 was produced in the rat anterior pituitary gland and hypothesized that RA was generated in the gland. Midkine (MK) is an RA-inducible growth factor, and MK production in the rat anterior pituitary gland was recently reported. However, the mechanism that regulates gene expression of MK in the pituitary gland has not been determined. To investigate regulation of MK production in the anterior pituitary gland, we analyzed changes in MK mRNA in cultured rat anterior pituitary cells. We identified MK-expressing cells by double-staining with in situ hybridization and immunohistochemical techniques for RALDH1. MK mRNA was expressed in RALDH1-producing cells in the anterior pituitary gland. Using isolated anterior pituitary cells of rats, we examined the effect of RA on gene expression of MK. Quantitative real-time PCR revealed that 72 h exposure to a concentration of 10^-6 M of retinal and all-trans retinoic acid increased MK mRNA levels by about 2-fold. Moreover, the stimulatory effect of all-trans retinoic acid was mimicked by the RA receptor agonist Am80. This is the first report to show that RA is important in regulating MK expression in rat anterior pituitary gland.

Key words: Retinoic acid, Midkine, Retinaldehyde dehydrogenase, Retinoic acid receptor, Anterior pituitary gland

Retinoic acid (RA) is a signaling molecule in embryonic development and adult tissue. RA is generated from retinal by the retinaldehyde dehydrogenases (RALDHs) which include RALDH1, RALDH2, and RALDH3 and acts on neighboring cells in an autocrine or paracrine manner [1]. Our previous work revealed that RALDH1 is produced in folliculostellate (FS) cells and lactotrophs in the adult rat anterior pituitary gland [2, 3]. We recently reported that RA promotes growth hormone–releasing hormone receptor (Ghrh-r) and growth hormone secretagogue receptor (Ghs-r) gene expression in rat anterior pituitary cells. In addition, RA increased growth hormone–releasing hormone (GHRH)- and ghrelin-stimulated growth hormone (GH) release from cultured anterior pituitary cells [4]. These findings suggest that RA is generated in FS cells and lactotrophs and that it has a role as an autocrine and/or paracrine signaling molecule in the gland.

Midkine (MK) is a member of the newly described heparin-binding growth factors [5]. It was initially identified as a neurotrophic factor induced by RA [6]. MK promotes growth, survival, and migration of various cells and has roles in neurogenesis and epithelial mesenchymal interactions during organogenesis [7]. Our previous study showed that FS cells produce MK in the adult rat anterior pituitary gland [8]. Moreover, the MK receptor protein tyrosine phosphatase zeta 1 (Ptpz1) is expressed in hormone-producing cells. However, the mechanism that regulates MK gene expression in the pituitary gland is not known. We sought to determine whether MK mRNA transcription in anterior pituitary cells is affected by RA. In this study, we first identified RALDH1 and MK gene expression in the anterior pituitary gland by in situ hybridization and immunohistochemistry. Then, we examined the effects of retinal, RA, and retinoic acid receptor (RAR) agonists on MK expression in a primary monolayer culture of anterior pituitary cells.
Materials and Methods

Animals
Male Wistar rats were purchased from Japan SLC (Shizuoka, Japan). Rats aged 9–10 weeks were housed in a temperature-controlled room (22 ± 1°C) with a 12-h light/12-h dark cycle and illumination from 0700 h to 1900 h and were given ad libitum access to conventional food and water. All animals were treated in accordance with the Guidelines for Animal Experimentation of Jichi Medical University, which is based on the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences, USA.

In situ hybridization and immunohistochemistry
In situ hybridization was performed with digoxigenin (DIG)-labeled cRNA probes, as described in our previous report [9]. Antisense and sense cRNA probes of rat MK were synthesized by in vitro transcription with DIG RNA Labeling Mix (Roche Diagnostics, Penzberg, Germany) [10]. Visualization of MK mRNA was performed with alkali phosphatase–conjugated anti-DIG antibody (Roche Diagnostics). For double-staining, after detection of MK mRNA by in situ hybridization, the section was immunostained with an antibody for rat RALDH1 [3], as described in our previous report [2]. The ABC method (Vector Laboratories, Burlingame, CA, USA) was used, with 3,3'-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) as the substrate.

Real-time PCR and reverse transcription (RT)-PCR
Quantitative real-time PCR and RT-PCR was performed as described in our previous report [8, 13]. The primers used to amplify cDNA fragments were listed in Table 1. For normalization, we also quantified β-actin. Relative gene expression was calculated by comparing the cycle times for each target PCR. Cycle threshold values were converted to relative gene expression levels with the 2^-(ΔCt sample - ΔCt control) method. In RT-PCR analysis, PCR products were run on 2% agarose gel and visualized with ethidium bromide.

Statistical analysis
The data were presented as means ± SEM for independent or group experiments repeated at least four times. The Dunnett test was used for statistical analysis. Differences between groups were considered statistically significant when the p value was less than 0.05.

Primary culture of anterior pituitary cells
Anterior pituitary cells of rats were dispersed as described previously [4]. The cells (5 × 10^5 cells) were seeded in 24-well plates and then incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were then exposed to retinal (Sigma-Aldrich, St. Louis, MO, USA), all-trans retinoic acid (ATRA; Sigma-Aldrich), Am80 [11], or PA024 [12] at predetermined concentrations and durations. Am80 and PA024 were kindly provided by Dr. Hiroyuki Kagechika (School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, Japan).

Table 1: List of primers used for the real-time PCR and RT-PCR

| Genes | Primer sequence (5’-3’) | Product size (bp) | GenBank accession number |
|-------|--------------------------|-------------------|-------------------------|
| MK    | Forward: TGGAGCCGACTGCAAATAC  
Reverse: TGTACCGAGCCTTTCAGG | 98 | AF315950 |
| β-actin | Forward: TGGCCACACACTTCTACATGAC  
Reverse: GGGTCATCTTTTCACGGTTGG | 106 | NM_031144 |
| Rar-a | Forward: GTGTCACCAGGACAAGCACT  
Reverse: GGTCACATGTTCACGTTG | 610 | NM_031528 |
| Rar-b | Forward: GGACCTTTGAGGGAACACTACAA  
Reverse: TCTCTGGCATGCATCTGAC | 622 | NM_031529 |
| Rar-g | Forward: CCACCAATAATGACATCAAG  
Reverse: GCTTCATCTCCTACGGAAC | 622 | NM_001135249 |
| Rxr-a | Forward: GCCTCACCAGAAGCATCCGCAAG  
Reverse: GCCTCATCTCCTACGGAAC | 559 | NM_012805 |
| Rxr-b | Forward: GGAACAGAAGAGCCAGACGAA  
Reverse: CAGGTGTCATGACAGACTG | 589 | NM_206849 |
| Rxr-g | Forward: CATCTGTGGGGGACAGAATCC  
Reverse: ATGGCATCCTGGACAGAACC | 555 | NM_031765 |
Results and Discussion

RA is a small lipophilic molecule that is important in cell growth, differentiation, and organogenesis. RA is synthesized from retinal by the RALDH family [1]. This is the first study to show that MK mRNA is expressed in RALDH1-producing cells, as indicated by in situ hybridization and immunohistochemistry (Fig. 1). RALDH1-immunopositive cells were located in the anterior lobe (Fig. 1b, f). MK-expressing cells were small and frequently aggregated in small clusters (Fig. 1c, g). We previously detected MK mRNA in FS cells [8]. In the present study, MK mRNA was detected in some RALDH1-immunopositive cells (Fig. 1d, h). Our previous study demonstrated that RALDH1-producing cells are FS cells and lactotrophs [2]. Taken together, these findings suggest a close relationship between expression of RALDH1 and MK in FS cells.

Fig. 1 Identification of RALDH1 in MK-expressing cells

Hematoxylin and eosin staining of a cryosection of rat pituitary gland (a, e): anterior lobe (AL), intermediate lobe (IL), and posterior lobe (PL). Immunohistochemistry of RALDH1 (b, f). In situ hybridization of MK mRNA (c, g). Double-staining of MK mRNA detected by in situ hybridization (blue) and RALDH1 protein detected by immunohistochemistry (brown) (d, h). The magnified images of AL in (a), (b), (c), and (d) are shown in (e), (f), (g), and (h), respectively. Cells exhibiting both an MK mRNA signal and RALDH1 immunoreactivity are indicated by arrowheads (h). Bars (a-d) 100 µm, (e-h) 10 µm.
To complement the above morphological studies, we used quantitative real-time PCR analysis to determine whether retinal or ATRA affects MK mRNA transcription in isolated cells from the adult rat anterior pituitary gland. MK gene expression was significantly increased by both retinal and ATRA treatment (Fig. 2). MK expression after exposure to $10^{-6}$ M of retinal or ATRA was 1.8 ($\pm 0.20$)- and 2.1 ($\pm 0.45$)-fold that of control (Fig. 2). Rat RALDH1 catalyzes oxidation of all-trans-retinal ($K_m = 9.8 \mu M$) to ATRA [14]. ATRA controls gene transcription via the nuclear receptor RAR [15]; however, retinal does not substantially bind RAR [16]. Our present findings suggest that locally generated RA induces MK gene expression in FS cells in an autocrine/paracrine manner.

Next, we examined time- and dose-dependent effects of ATRA on MK gene expression in isolated anterior pituitary cells. ATRA gradually increased MK mRNA transcription during the period from 24 to 72 h (Fig. 3). MK mRNA transcription levels after exposure to ATRA ($10^{-6}$ M) for 24, 48, and 72 h were 1.7 ($\pm 0.17$)-, 2.0 ($\pm 0.13$)- and 2.1 ($\pm 0.29$)-fold that of control. Pituitary cells were incubated with increasing doses of ATRA ($10^{-8}$ to $10^{-6}$ M) for 72 h (Fig. 4). ATRA ($10^{-6}$ M) increased MK mRNA transcription levels by 2.1 ($\pm 0.26$)-fold. These results are consistent with the findings of previous studies, which reported that RA induced MK expression in mouse and human embryonal carcinoma cell lines [6, 17]. Minegishi et al. used Northern blot analysis to study regulation of MK expression by RA in cultured rat ovarian granulosa cells [18] and found that MK mRNA transcription after exposure to RA ($3 \times 10^{-7}$ M) for 24 h was increased by 2-fold. These and our findings indicate that RA plays a role as a general stimulator of MK.

![Fig. 2](image2.png)

**Fig. 2** Effects of retinal and ATRA on MK gene expression
Isolated anterior pituitary cells were treated with retinal, ATRA ($10^{-6}$ M), or vehicle for 72 h. MK mRNA levels were measured by real-time PCR and are shown as ratios of control values (vehicle). The data are means $\pm$ SEM ($n = 4$). **$p < 0.01$ vs. control.

![Fig. 3](image3.png)

**Fig. 3** Time course effect of ATRA on MK mRNA levels
Isolated anterior pituitary cells were treated with ATRA ($10^{-6}$ M) for 0, 24, 48, or 72 h. MK mRNA levels were measured by real-time PCR and are shown as ratios of control values (0 h). The data are means $\pm$ SEM ($n = 4$). *$p < 0.05$, **$p < 0.01$ vs. control.

![Fig. 4](image4.png)

**Fig. 4** Dose–response effect of ATRA on MK mRNA transcription
Isolated anterior pituitary cells were treated with different doses of ATRA ($10^{-8}$ M to $10^{-6}$ M) or vehicle as control for 72 h. MK mRNA levels were measured by real-time PCR and are shown as ratios of control values. The data are means $\pm$ SEM ($n = 4$). **$p < 0.01$ vs. control.
RA is a ligand for two families of nuclear receptors, the RARs and retinoid X receptors (RXRs), and the RA–RAR/RXR complex binds to a sequence referred to as the retinoic acid response element (RARE) and regulates transcription of target genes [15]. We examined the effects of the synthetic RARα and β agonist Am80 and the RXR pan-agonist PA024 on MK gene expression. As shown in Fig. 5, MK mRNA transcription after exposure to Am80 was 2.0 (± 0.18)-fold that of control. Am80 treatment completely mimicked the effect of ATRA on MK mRNA transcription. In contrast, PA024 had no effect on MK mRNA transcription. To confirm the effect of RA, we demonstrated the expression of RARs and RXRs in the anterior pituitary gland by means of RT-PCR. Rar-a, Rar-b, Rar-g, Rxr-a, Rxr-b, and Rxr-g mRNAs were expressed in the gland of the rat (Fig. 6). Matsubara et al. reported that the 5′-flanking region of the mouse MK gene has an important RARE between nucleotides -1,006 and -794 [19]. This finding and our present results indicate that RA directly promotes MK gene expression via RAR.

In a previous study, we reported that the MK receptor Ptprz1 was expressed in somatotrophs and corticotrophs in rat anterior pituitary gland [8]. We hypothesized that MK produced in FS cells acts locally on these hormone-producing cells via PTPRZ1 in the anterior pituitary. We also reported that RA increased Ghrh-r and Ghs-r gene expression and promoted GHRH- and ghrelin-induced GH release from isolated rat anterior pituitary cells [4]. Past and present evidence suggests that RA and MK function as autocrine and paracrine signaling molecules in the anterior pituitary gland. Future studies should attempt to clarify the roles of RA and MK in cell–cell interaction in the gland.

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Disclosure

The authors have no conflict of interest that might prejudice the impartiality of this research.

Fig. 5  Effects of RAR and RXR agonists on MK mRNA transcription

Isolated anterior pituitary cells were treated with the RARα and β agonist Am80, the RXR pan-agonist PA024, or ATRA (10⁻⁶ M) for 72 h. MK mRNA levels were measured by real-time PCR and are shown as ratios of control values. The data are means ± SEM (n = 4). ** p < 0.01 vs. control.

Fig. 6  Expression of RARs and RXRs in anterior pituitary gland of the rat

Expression of RARs (Rar-a, Rar-b, Rar-g) and RXRs (Rxr-a, Rxr-b, Rxr-g) in anterior pituitary gland was determined by RT-PCR. M: 100 bp DNA ladder maker.

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