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Regulation of hyaluronate metabolism by progesterone in cultured fibroblasts from the human uterine cervix

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Abstract The effects of progesterone, dehydroepiandrosterone sulfate and 17β-estradiol on the synthesis and degradation of hyaluronate were investigated using human uterine cervix fibroblasts. The cells were incubated with [^{3}H]glucosamine in the presence of the hormones and then [^{3}H]hyaluronate was isolated from the medium. The changes in the radioactivity of [^{3}H]hyaluronate showed that progesterone suppressed hyaluronate synthesis by 22% of the control levels, while dehydroepiandrosterone sulfate and 17β-estradiol enhanced it by 22% and 12% of the control levels, respectively. Furthermore, progesterone induced degradation of high-molecular-weight [^{14}C]hyaluronate into low-molecular-weight hyaluronate ($M_r \approx 40\,000$). These results suggest that in cultured fibroblasts from the human uterine cervix progesterone converts hyaluronate metabolism from the synthesis phase to the degradation phase.

Key words: Hyaluronate; Human uterine cervix; Progesterone; Hyaluronate metabolism; Cultured fibroblast

1. Introduction

In human uterine cervix connective tissue, it has been reported that the amount of hyaluronate increases markedly at the last stage of pregnancy and quickly decreases after parturition [1–3]. However, the mechanism responsible for this rapid hyaluronate synthesis and degradation has not yet been clarified.

Recently, Tanaka et al. [4] found that hyaluronidase activity was absent from human uterine cervix fibroblasts and that a hyaluronate-depolymerizing enzyme, endo-β-*N*-acetylglucosaminidase [5], was activated in these cells by progesterone. In the present study, in order to elucidate hyaluronate metabolism in the uterine cervix, the effects of hormones, especially progesterone, on the synthesis and degradation of hyaluronate were investigated using cultured fibroblasts derived from the human uterine cervix. The results showed that progesterone suppresses hyaluronate synthesis and, at the same time, converts hyaluronate metabolism from the synthesis phase to the degradation phase in these cultured cells.

2. Materials and methods

2.1. Chemicals

D-[6-^{3}H]Glucosamine (specific activity 40 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA, USA). [^{14}C]Hyaluronate (M_r=1.86×10^6, specific activity 3.14 μCi/mg) and non-radioactive hyaluronate (M_r=1.90×10^6, 1.0×10^5 and 4.0×10^4) were the same as described previously [4]. *Streptomyces* hyaluronidase was purchased from Seikagaku (Tokyo, Japan), and actinase E was obtained from Kaken Seiyaku (Tokyo, Japan). Dehydroepiandrosterone sulfate (DHAS), 17β-estradiol and progesterone were purchased from Sigma (St. Louis, MO, USA). All other reagents used were obtained from commercial sources.

2.2. Cell culture

Human uterine cervix and skin fibroblasts were cultured to confluence in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum in a humidified atmosphere of 5% CO_2/95% air at 37°C, as described previously [4]. Cells at the second to sixth passage were used for the following experiments. The passage number did not affect the responses to hormones and other chemical reagents.

2.3. Analysis of synthesized [^{3}H]hyaluronate

Uterine cervix fibroblasts were incubated in DMEM containing [^{3}H]glucosamine (2 μCi/ml) for 48 h, and a medium fraction was obtained as described by Nakamura et al. [5]. As incorporation of [^{3}H]glucosamine into the matrix and cell fractions was much smaller than that into the medium fraction in preliminary experiments, medium fractions were used in this investigation. Ethanol saturated with NaCl (4 vols.) was then added to the medium fraction and the mixture was cooled and centrifuged. The precipitate was incubated with actinase E in 50 mM Tris-HCl buffer (pH 8.0) overnight at 37°C. The reaction mixture was heated to 100°C for 3 min and then centrifuged. The supernatant was subjected to ethanol precipitation and the resulting precipitate was used as a [^{3}H]glycosaminoglycan fraction. This fraction was dissolved in 100 mM sodium acetate buffer (pH 5.0) and an aliquot was subjected directly to high-performance liquid chromatography (HPLC). The rest of the solution was subjected to HPLC after *Streptomyces* hyaluronidase digestion for 12 h at 37°C. The HPLC was carried out on a Shodex OHpak KB-805 column (0.8×30 cm) (Showa Denko, Tokyo, Japan) eluted with 0.2 M NaCl at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and their radioactivity was counted using a liquid scintillation counter. Non-radioactive hyaluronates (M_r=4.0×10^4, 1.0×10^5 and 1.90×10^6) and hyaluronate tetrasaccharides obtained by digestion with *Streptomyces* hyaluronidase were used as molecular weight standards.

For determination of [^{3}H]hyaluronate, [^{3}H]glycosaminoglycan fractions were obtained and digested with *Streptomyces* hyaluronidase as described above. Ethanol saturated with NaCl (4 vols.) was added to the reaction mixture, which was then centrifuged. The supernatant was collected and its radioactivity was measured. The radioactivities of the medium fractions were defined as the amount of high-molecular-weight hyaluronate.

2.4. Effects of hormones on [^{3}H]hyaluronate synthesis

Uterine cervix fibroblasts were preincubated at 37°C in DMEM containing hormones (DHAS, 1×10^{-6} M; 17β-estradiol, 1×10^{-7} M or progesterone, 1×10^{-7} M). After 12 h the medium was removed and the cells were then incubated with fresh medium containing [^{3}H]glucosamine (2 μCi/ml) and the same hormone for 48 h. The radioactivity of [^{3}H]hyaluronate in the medium fraction was determined. The concentration of these hormones in the medium was based

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Abbreviations: DHAS, dehydroepiandrosterone sulfate; DMEM, Dulbecco's modified Eagle medium; HPLC, high-performance liquid chromatography

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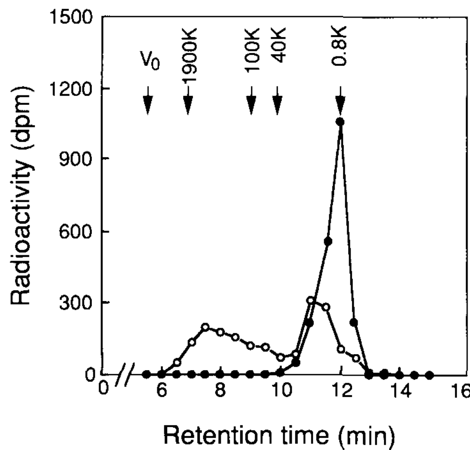

Fig. 1. HPLC of [^{3}H]hyaluronate synthesized by human uterine cervix fibroblasts. Human uterine cervix fibroblasts were incubated for 48 h in the presence of [^{3}H]glucosamine. The medium with (●) or without (○) *Streptomyces* hyaluronidase treatment was then applied to a Shodex OHpak KB-805 column. Arrows indicate the elution positions of standard hyaluronate ($M_r = 1.90 \times 10^6$, 1.0×10^5 and 4.0×10^4) and hyaluronate tetrasaccharides (0.8 K). V_0, void volume.

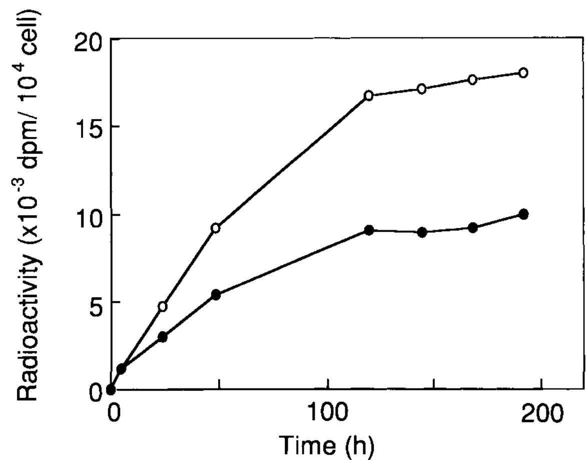

Fig. 2. Comparison of hyaluronate synthesis between human uterine cervix and skin fibroblasts. Incorporation of [^{3}H]glucosamine into [^{3}H]hyaluronate synthesis in cervix (○) and skin (●) fibroblasts was determined after incubation for 192 h. The radioactivity of [^{3}H]hyaluronate from the medium fraction was defined as the amount of [^{3}H]hyaluronate, which was expressed as hyaluronate per 10^4 cells after the end of incubation. Data represent the mean values of three experiments.

on their concentrations in the plasma of pregnant women at term [6,7].

3. Results

3.1. *Analysis of synthesized [^{3}H]hyaluronate*

Uterine cervix fibroblasts were incubated in medium containing [^{3}H]glucosamine for 48 h. The [^{3}H]glycosaminoglycan fraction obtained from the medium fraction was subjected to HPLC. Two peaks were observed, and the peak eluted in the higher-molecular-weight region (retention time 6–10 min) was digested completely by *Streptomyces* hyaluronidase (Fig. 1). These results indicate that the ^{3}H-labeled material of high molecular weight, which was synthesized by uterine cervix fibroblasts, was hyaluronate with an average molecular weight of about 1×10^6.

3.2. *Comparison of [^{3}H]hyaluronate synthesis of uterine cervix and skin fibroblasts*

Uterine cervix and skin fibroblasts were incubated in medium containing [^{3}H]glucosamine and then hyaluronate synthesis was compared. The incorporation of [^{3}H]glucosamine into hyaluronate by these fibroblasts increased linearly with time, almost reaching a plateau after 120 h. After this time, the incorporation of [^{3}H]glucosamine into hyaluronate by uterine cervix fibroblasts was about twice that by skin fibroblasts (Fig. 2).

3.3. *Effects of hormones on [^{3}H]hyaluronate synthesis*

Uterine cervix and skin fibroblasts were preincubated in medium containing hormones (DHAS, 17β-estradiol or progesterone) for 12 h. The medium was removed and the incubation was continued with fresh medium containing [^{3}H]glucosamine and the same hormone. After 48 h, the radioactivities of hyaluronate synthesized by these cells were determined. In skin fibroblasts, the radioactivities of hyaluronate showed no difference between hormone-treated cells and control cells (Fig. 3A). On the other hand, in the presence of 10^{-7} M progesterone the radioactivity of hyaluronate in uterine cervix fibroblasts was decreased by 22% of the control level. In contrast, in the presence of 10^{-6} M DHAS and 10^{-7} M 17β-estradiol, it was increased by about 22% and 12% of the control level, respectively (Fig. 3B).

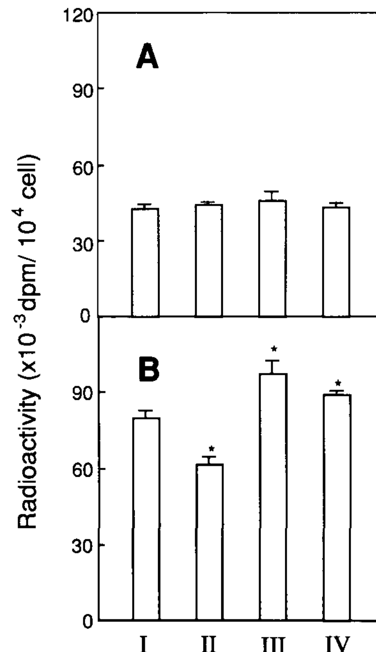

Fig. 3. Effects of hormones on [^{3}H]hyaluronate synthesis in human skin and uterine cervix fibroblasts. Human skin (A) and uterine cervix (B) fibroblasts were incubated with [^{3}H]glucosamine and hormones. After 48 h, the incorporation of [^{3}H]glucosamine into hyaluronate was determined as described in the text. [^{3}H]Hyaluronate synthesis was expressed as hyaluronate per 10^4 cells after the end of incubation. Data are means ± S.D. of three experiments. *, significantly different from control ($P < 0.01$). I, control without hormones; II, progesterone (1×10^{-7} M); III, dehydroepiandrosterone sulfate (1×10^{-6} M); IV, 17β-estradiol (1×10^{-7} M).

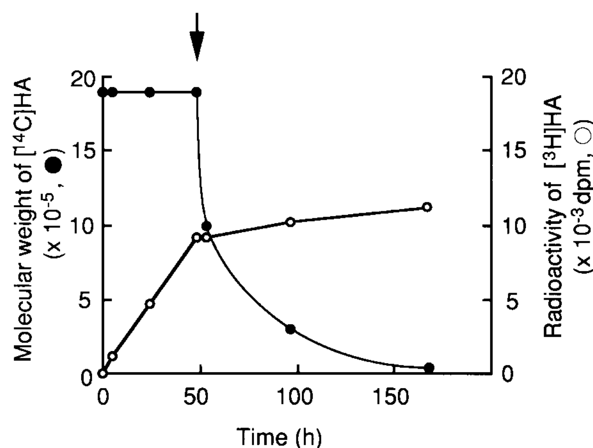

Fig. 4. Effects of progesterone on hyaluronate metabolism in uterine cervix fibroblasts. Human uterine cervix fibroblasts were incubated with both [^{3}H]glucosamine and [^{14}C]hyaluronate for 48 h, after which 10^{-7} M progesterone was added to the medium. After incubation with progesterone for 5, 48 and 120 h, a portion of the medium was recovered and the incorporation of [^{3}H]glucosamine into hyaluronate (○) and the molecular weight of [^{14}C]hyaluronate (●) were measured. The arrow indicates the time of addition of progesterone to the medium.

3.4. *Effects of progesterone on hyaluronate synthesis and degradation of uterine cervix fibroblasts*

Uterine cervix fibroblasts were incubated in medium containing both [^{3}H]glucosamine (2 μCi/ml) and [^{14}C]hyaluronate (M_r=1.86×10^6, 0.09 μCi/ml, 14.3 μg/ml). After 48 h, 10^{-7} M progesterone was added to the medium and the incubation was continued. A portion of medium was recovered during incubation with progesterone, and the incorporation of [^{3}H]glucosamine into hyaluronate and alterations in the chain length of [^{14}C]hyaluronate were measured simultaneously. During incubation without progesterone, the incorporation of [^{3}H]glucosamine into hyaluronate increased in a time-dependent manner and the [^{14}C]hyaluronate was not depolymerized (Fig. 4). After progesterone had been added to the medium, the incorporation of [^{3}H]glucosamine into hyaluronate was suppressed and reached a plateau. At the same time, it was observed that [^{14}C]hyaluronate began to be depolymerized. After incubation with progesterone for 120 h, the hyaluronate had been depolymerized to a low-molecular-weight fragment ($M_r \approx 40\,000$) (Fig. 4).

4. Discussion

Hyaluronate is one of the primary constituents of the extracellular matrix of the human uterine cervix. It has been reported that during cervix ripening the amount of hyaluronate increases to about 10 times the non-pregnant level, and decreases rapidly back to the non-pregnant level after parturition [1,3]. These dramatic changes suggest that hyaluronate plays an important role in the regulation of cervix function during parturition. Although stimulation of hyaluronate production by substances such as prostaglandin E_2, prostaglandin $F_{2\alpha}$ [8] and interleukin-1α [9] has been reported, the mechanisms of hyaluronate metabolism and its regulation in the cervix are unclear.

Comparison of hyaluronate in fibroblasts from the uterine cervix and skin showed that the former synthesized about twice as much hyaluronate as the latter (Fig. 2). The molecular weight of the hyaluronate synthesized by cervix fibroblasts was estimated to be about 1×10^6 by gel-filtration HPLC (Fig. 1). In the cervix fibroblasts, hyaluronate synthesis was suppressed by progesterone, and stimulated by DHAS and 17β-estradiol, respectively (Fig. 3B). In the contrast, these hormones were scarcely effective on hyaluronate synthesis in skin fibroblasts (Fig. 3A). These results suggest that the regulation of hyaluronate synthesis in uterine cervix fibroblasts is under hormonal control.

Progesterone (10^{-7} M) was added to the medium after preincubation of uterine cervix fibroblasts in the presence of both [^{3}H]glucosamine and [^{14}C]hyaluronate, and then incorporation of [^{3}H]glucosamine into hyaluronate and changes in the chain length of [^{14}C]hyaluronate were monitored. As a result, the synthesis of [^{3}H]hyaluronate was suppressed and [^{14}C]hyaluronate began to depolymerize to a low molecular weight almost immediately after the addition of progesterone (Fig. 4). These results indicate that progesterone induces rapid conversion of hyaluronate metabolism from the synthesis phase to the degradation phase.

In our previous study [4], the progesterone antagonist RU38486 inhibited effectively the progesterone-induced depolymerization of hyaluronate in a dose-dependent manner. Therefore, it may be reasonable to consider that progesterone binds to its receptors on cervix fibroblasts [10], leading not only to the suppression of hyaluronate synthesis but also to the progression of hyaluronate degradation by inducing an endo-β-*N*-acetylglucosaminidase, which differs from hyaluronidase [5]. It has been reported that progesterone in the uterine cervix participates in the maintenance of firm connective tissue [11], inhibits uterine contractions [12], and suppresses the production of a neutrophil chemotactic factor/interleukin-8 [13]. These results suggest that progesterone helps to keep the uterine cervix firm and avoids preterm delivery by suppressing hyaluronate synthesis and collagenolysis [11]. It is likely that further study of the regulation of hyaluronate metabolism by progesterone will help to elucidate the mechanisms of cervical changes during parturition.

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