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Hyaluronate depolymerization activity induced by progesterone in cultured fibroblasts derived from human uterine cervix

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Abstract

High-molecular-weight [^{14}C]hyaluronate was incubated with cultured fibroblasts from human uterine cervix and skin, and then the depolymerization of the hyaluronate was investigated. [^{14}C]Hyaluronate in the medium of skin fibroblasts was depolymerized into a constant molecular weight (M_r about 40,000), whereas that of cervix fibroblasts was not depolymerized, irrespective of incubation period. However, when progesterone was added to the medium of cervix fibroblasts, hyaluronate was depolymerized to the same extent as that in skin fibroblasts. The reducing terminal sugar of the depolymerized hyaluronate was *N*-acetylglucosamine. These results suggest that a hyaluronate-depolymerizing enzyme, endo-β-*N*-acetylglucosaminidase, was induced by progesterone in cultured fibroblasts derived from human uterine cervix.

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

1. Introduction

Hyaluronate is widely distributed in tissue as one of the major components of the extracellular matrix. Its catabolism seems to occur through cleavage of the internal bonds of the sugar chain by hyaluronidase (endo-β-*N*-acetylhexosaminidase). The resulting oligosaccharides are then depolymerized successively by exo-β-glucuronidase and exo-β-*N*-acetylhexosaminidase from the nonreducing terminal [1], and at the final step, the resulting disaccharide is completely depolymerized to monosaccharide by disaccharide-depolymerizing exo-β-glucuronidase [2].

With regard to the mechanism of hyaluronate depolymerization in skin, Arbogast et al. demonstrated an absence of hyaluronidase in cultured human skin fibroblasts [3]. Nakamura et al. recently reported that high-molecular-weight hyaluronate was depolymerized to a constant molecular weight (M_r = 40,000) in the culture medium, and that the reducing terminal of the depolymerized molecule was a newly exposed *N*-acetylglucosamine [4]. Thus the presence of a hyaluronate-depolymerizing enzyme, endo-β-*N*-acetylglucosaminidase, was demonstrated in cultured human skin fibroblasts. Since then, hyaluronate-depolymerizing enzymes having a similar mechanism of action have been reported by other investigators [5,6].

In human uterine cervical connective tissue, it has been reported that the amount of hyaluronate increases markedly at the last stage of pregnancy and quickly decreases after parturition [7–9]. However, the mechanism responsible for the rapid depolymerization of hyaluronate after parturition in human uterine cervix has not yet been clarified. In order to resolve this issue, changes in the molecular weight of hyaluronate during fibroblast incubation and the effects of hormones on the hyaluronate depolymerization by cultured human uterine cervix fibroblasts were investigated. It was found that hyaluronate depolymerization activity corresponding to that reported by Nakamura et al. [4] was induced by progesterone in the cultured fibroblasts derived from human uterine cervix.

2. Materials and methods

2.1. Chemicals

[^{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 kindly supplied by Denki Kagaku Kogyo (Tokyo, Japan). [^{14}C]Hyaluronate was synthesized by *Streptococcus equi* in medium containing D-[U-^{14}C]glucose. Sodium [^{3}H]borohydride (specific activity 100 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) and Dulbecco's calcium- and magnesium-free phosphate-buffered saline (CMF-PBS) were purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) and penicillin- streptomycin solution (penicillin 5,000 units/ml and streptomycin 5,000 μg/ml) were purchased from Gibco (Grand Island, NY, USA). *Streptomyces* hyaluronidase was purchased from Seikagaku Kogyo (Tokyo, Japan). Dexamethasone (Dex), dehydroepiandrosterone sulfate (DHAS), estra-

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Abbreviations: Dex, dexamethasone; DHAS, dehydroepiandrosterone sulfate; PGF_{2a}, prostaglandin F_{2a}; PGE_2, prostaglandin E_2; IL-1α, interleukin 1α; DMEM, Dulbecco's modified Eagle medium; CMF-PBS, Dulbecco's calcium- and magnesium-free phosphate-buffered saline; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography.

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diol, progesterone, prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were purchased from Sigma Chemical (St. Louis, MO, USA). Recombinant human interleukin-1α (IL-1α, specific activity, 1×10^8 U/mg) was purchased from Genzyme (Boston, MA, USA). The progesterone antagonist RU38486 was kindly supplied by Roussel-Uclaf (Paris, France). Other reagents and chemicals were obtained from commercial sources.

2.2. Cell culture

A specimen of normal human uterine cervix was collected after a total hysterectomy for uterine myoma at Hirosaki University Hospital and Hirosaki City Hospital after informed consent had been obtained. Human skin was obtained from normal regions around a surgical wound at the time of scar removal. These tissue samples were cut into small pieces, which were then incubated in DMEM supplemented with FBS (10% v/v) and antibiotics at 37° in a humidified atmosphere of 5% CO2/95% air on 35-mm plastic dishes (Corning, Corning, NY, USA). After the fibroblasts had grown to confluency, they were used at up to fifth passage for this study.

2.3. Depolymerization of [^{14}C]hyaluronate

Human uterine cervix and skin fibroblasts at confluency were incubated in dishes containing medium supplemented with [^{14}C]hyaluronate (0.045 μCi, 14.3 μg/ml). After 5 h, 48 h and 120 h, the medium was recovered and each sample subjected directly to HPLC using a Shodex OHpak KB-805 column (0.8 × 30 cm, Showa Denko, Tokyo, Japan) eluted with 0.2 M NaCl at 30°. The flow rate was 1 ml/min, and 0.5-ml fractions were collected using a fraction collector. The radioactivity of each fraction was measured by a liquid scintillation counter (LSC-3500, Aloka, Tokyo, Japan). Non-radioactive hyaluronate ($M_r = 1.90 \times 10^6$, 1.0×10^5 and 4.0×10^4) and hyaluronate tetrasaccharides obtained by digestion with *Streptomyces* hyaluronidase were used as molecular weight standards.

2.4. Effects of hormones and IL-1α on [^{14}C]hyaluronate depolymerization

The effects of hormones and IL-1α were investigated. First, cervix and skin fibroblasts were preincubated in medium containing hormones or IL-1α. After preincubation for 12 h, the medium was removed and each cell layer was washed 3 times with CMF-PBS. Then, incubation was continued with fresh medium containing [^{14}C]hyaluronate and the same hormones or IL-1α. The medium was recovered after 5, 48, or 120 h and subjected to HPLC. Hormones that are increased in the plasma of pregnant women, and also IL-1α, were added to the medium in the form of an ethanol solution. The concentration of Dex, estradiol and progesterone (1×10^{-7} M, respectively) was based on the plasma concentration in pregnant women at term [10]. DHAS, PGE_2 and $PGF_{2\alpha}$ (1×10^{-6} M, respectively) were used at theconcentration effective for ripening of the uterine cervix [11,12]. IL-1α (1 ng/ml) was used at the concentration optimal for effective synthesis of hyaluronate [13].

2.5. Identification of the reducing terminal sugar

The reducing terminal sugar of the depolymerized hyal- uronate was investigated as described previously [14]. Cervix fibroblasts were preincubated in medium containing 10^{-7} M progesterone. After preincubation for 12 h, the medium was removed and incubation was continued with fresh medium containing non-radioactive hyaluronate ($M_r = 1.90 \times 10^6$, 1 mg/ml) and 10^{-7} M progesterone. The medium was recovered after 120 h and the depolymerized hyaluronate (M_r about 40,000) was tritiated and reduced using NaB^3H_4, followed by acid hydrolysis. The product was subjected to paper chromatography, and the radioactivity on the paper was determined.

3. Results

3.1. Depolymerization of [^{14}C]hyaluronate in the incubation medium

Human uterine cervix and skin fibroblasts were incubated in medium containing [^{14}C]hyaluronate for 5, 48 and 120 h. After each incubation period, the medium was recovered and subjected directly to HPLC. The high-molecular-weight hyaluronate in the medium of skin fibroblasts was found to be depolymerized as the incubation period was prolonged, and after 120 h, the molecular weight of the main depolymerized hyaluronate was estimated to be about 40,000 by HPLC, although the depolymerized hyaluronate showed a dispersed elution profile (Fig. 1A). On the other hand, in the medium of cervix fibroblasts, the high-molecular-weight hyaluronate was not depolymerized at all (Fig. 1B).

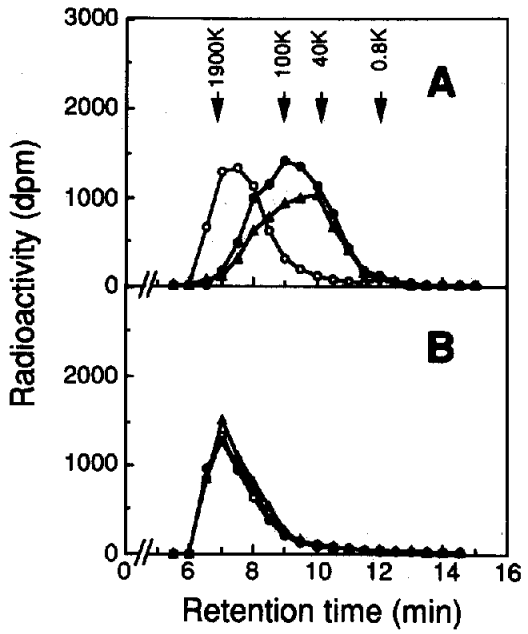

Fig. 1. HPLC of [^{14}C]hyaluronate in the incubation medium. Human skin (A) and uterine cervix (B) fibroblasts were incubated in medium containing [^{14}C]hyaluronate for 5 h (○), 48 h (●) and 120 h (△). After incubation, the medium was subjected directly to HPLC (column, Shodex OHpak KB-805). Arrows indicate the elution positions of standard hyaluronate ($M_r = 1.90 \times 10^6$, 1.0×10^5, 4.0×10^4 and hyaluronate tetrasaccharide (0.8K)).

3.2. Effects of hormones and IL-1α on [^{14}C]hyaluronate depolymerization

In cervix fibroblasts, the effects of hormones and IL-1α were then investigated. First, cervix fibroblasts were preincubated in medium containing hormones or IL-1α. After preincubation for 12 h, the medium was removed and incubation was continued with fresh medium containing [^{14}C]hyaluronate and the same hormones or IL-1α. The medium was recovered after 5, 48, or 120 h and subjected to HPLC. When DHAS was added to the culture medium, the high-molecular-weight hyaluronate was not depolymerized, irrespective of the incubation period (Fig. 2A). The same results were observed after addition of other hormones and IL-1α (data not shown). Only when progesterone was added was the high-molecular-weight hyaluronate depolymerized to low molecular weight (M_r about 40,000) (Fig. 2B).

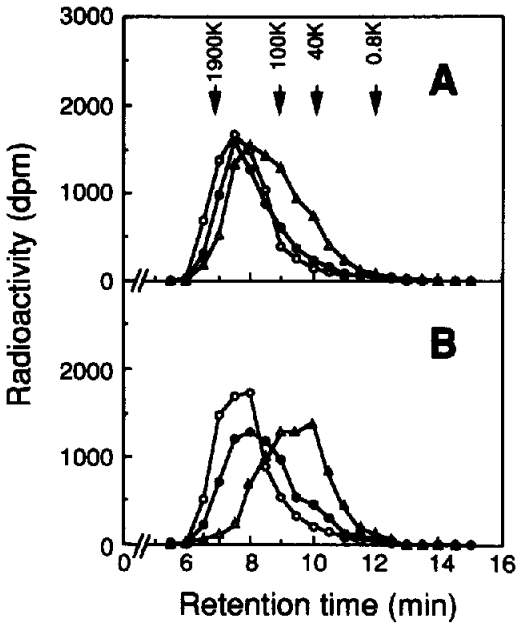

Fig. 2. Effects of hormones on [^{14}C]hyaluronate depolymerization in human uterine cervix fibroblasts. Cervix fibroblasts was incubated with [^{14}C]hyaluronate and hormones or IL-1α for 5 h (○), 48 h (●) and 120 h (△). After incubation, the medium was subjected directly to HPLC. A, DHAS; B, progesterone. Arrows indicate the elution positions of molecular weight standards.

3.3. *Effects of progesterone on [^{14}C]hyaluronate depolymerization*

The stimulatory effects of various concentrations of progesterone on hyaluronate depolymerization by cervix fibroblasts were then studied. After incubation with progesterone for 120 h, the medium was subjected to HPLC. As shown in Fig. 3A, the hyaluronate depolymerization described above was marked when 10^{-6}–10^{-7} M progesterone was present. However, when the concentration of progesterone was below 10^{-9} M, no hyaluronate depolymerization was observed.

To investigate whether the depolymerization of hyaluronate was indeed stimulated by progesterone, various concentrations of the progesterone antagonist RU38486 [15] were added to the medium containing 10–7 M progesterone. After 120 h, the medium was recovered and the depolymerization of hyaluronate was estimated by HPLC. RU38486 added to the medium at 10–11 M with 10^{-7} M progesterone did not inhibit depolymerization. However, as the concentration of RU38486 increased, the depolymerization of hyaluronate began to be inhibited, and was completely abolished at a concentration of 10^{-5} M (Fig. 3B).

On the other hand, when some hormones (Dex, DHAS, estradiol, PGE_2, $PGF_{2\alpha}$ and progesterone) and IL-1α were added to the culture medium of the skin fibroblasts, the molecular weight of the depolymerized products remained at about 40,000, and when 10^{-7} M RU38486 was added to the medium, hyaluronate depolymerization was not inhibited (data not shown).

3.4. *Identification of the reducing terminal sugar*

The reducing terminal sugar of the hyaluronate which had been depolymerized by addition of 10^{-7} M progesterone was then investigated by paper chromatography after reduction of the non-radioactive hyaluronate with NaB^3H_4. The reducing terminal sugar was identified as *N*-acetylglucosamine (data not shown).

4. Discussion

Hyaluronate is one of the primary constituents of the extracellular matrix of the human uterine cervix, and it has been reported that the amount of hyaluronate increases to about 10 times the regular level at the onset of labor and decreases quickly to the former level after parturition [7,9]. This dramatic change appears to indicate that hyaluronate plays an important role in the regulation of uterine cervical function during parturition. However, the mechanisms of hyaluronate depolymerization and its regulation in the uterine cervix are not yet clear.

In the present study, in order to elucidate the mechanism of hyaluronate depolymerization in cervix fibro-

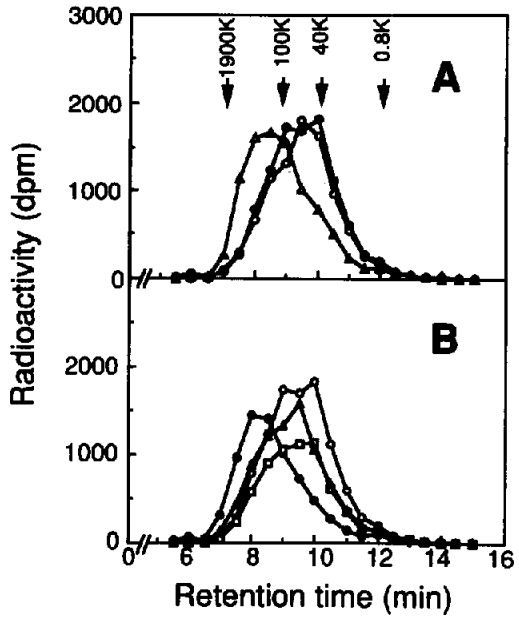

Fig. 3. Effects of progesterone on [^{14}C]hyaluronate depolymerization in human uterine cervix fibroblasts. (A) [^{14}C]Hyaluronate depolymerization in human cervix fibroblasts was studied using various concentrations of progesterone. ○, 10^{-6} M; ●, 10^{-7} M; △, 10^{-9} M. (B) Various concentrations of progesterone antagonist, RU38486, were added to the medium along with 10^{-7} M progesterone. ○, 10^{-7} M progesterone (control); ●, 10^{-5} M RU38486 with 10^{-7} M progesterone; △, 10^{-9} M RU38486 with 10^{-7} M progesterone; □,10^{-11} M RU38486 with 10^{-7} M progesterone. Arrows indicate the elution positions of molecular weight standards.

blasts, the activity of hyaluronidase was measured by the method of Nakamura et al. using fluorogenic hyaluronate as a substrate [16]. Hyaluronidase degrades hyaluronate into oligosaccharides (tetra- and hexasaccharides). However, it was found that oligosaccharides such as tetra- and hexasaccharides were undetectable in both the conditioned medium and the cell layer (data not shown). Therefore, the depolymerization of radioactive [^{14}C]hyaluronate was investigated in medium derived from cervix and skin fibroblasts. It was found that the hyaluronate of high molecular weight in the medium of skin fibroblasts was depolymerized to units of M_r about 40,000, corresponding to the previous report of Nakamura et al. [4] (Fig. 1A).

In cervix fibroblasts, only when progesterone was added to the medium was hyaluronate depolymerization observed, and the M_r of the main depolymerization product was estimated to be about 40,000 by HPLC (Fig. 2B). However, prolongation of the incubation time did not produce oligosaccharides, as shown by hyaluronidase digestion. Furthermore, the progesterone antagonist RU38486 effectively inhibited the progesterone-induced depolymerization of hyaluronate in a dose-dependent manner, as shown in Fig. 3B. These results suggest that the depolymerization of hyaluronate is induced by progesterone and may be regulated through the progesterone receptor. Furthermore, the reducing terminal sugar of the depolymerized hyaluronate was identified as *N*-acetylglucosamine. Therefore, it seems that progesterone binds to progesterone receptors in cervix fibroblasts and induces a novel endo-*β*-*N*-acetylglucosaminidase, which is different from the hyaluronidase. The mechanism responsible for this induction is still not clear, but the enzyme may be similar to the endo-*β*-*N*-acetylglucosaminidase reported by Nakamura et al. [4]. It is likely that further study of the depolymerizing enzyme induced by progesterone will help to elucidate the mechanisms of hyaluronate depolymerization and its regulation in the connective tissue of the human uterine cervix after parturition.

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