Endogenous Hyaluronan: A Cytokine-Like Factor Present in Rabbit Uterine Cervix during Pregnancy

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The effects of endogenous hyaluronan on cervical ripening during pregnancy were examined in rabbits. Hyaluronan of approximately 620 kilodalton (kDa) was detected in the uterine cervix on the 25th and 29th days of pregnancy, while it was not detected in cervix of non-pregnant animals. In addition, low-molecular-weight (less than 191 kDa) hyaluronan was present in cervix at the 29th day of pregnancy. Hyaluronan level in the cervix was lower on the 29th day than on the 25th day of pregnancy, whereas that in serum was significantly higher on the 29th day than on the 25th day of pregnancy. To clarify the physiological functions of endogenous hyaluronan, the effects of hyaluronan (HA600—700), which had approximately equal to endogenous hyaluronan, on uterine cervical tissues and uterine cervical fibroblasts were examined. Rabbits at the 23rd day of pregnancy were administered a vaginal suppository of HA600—700 (20 mg) daily for three days. Promotion of cervical ripening was observed, as well as detachment of collagen fiber bundles, and a reduced density of collagen fiber distribution. Total collagenolytic activity was increased significantly by HA600—700 (1.0 mg/ml) treatment in cultured uterine cervical explants of pregnant rabbits as compared with the untreated group. Moreover, very similar effects of HA600—700 treatment (0.1, 1.0 mg/ml) were observed in cultured uterine cervical fibroblasts. Further, in tissue cultures, but not cell cultures, of pregnant rabbit cervix, prostaglandin E2 (PGE2) production was enhanced by HA600—700 treatment. Therefore, it appears that endogenous hyaluronan is closely concerned with cervical ripening and dilatation in uterine cervix of pregnant rabbits.

Key words endogenous hyaluronan; cytokine; cervical ripening; uterine cervix; collagenolytic activity; prostaglandin E2 (PGE2)

The uterine cervix is a typical connective tissue, and consists mainly of collagen, hyaluronan, and proteoglycan.1) During gestation, it must close firmly to retain the fetus in the uterus. It undergoes ripening and dilatation at term, forming a parturient canal so that delivery can occur. During ripening, there are increases of collagen degradation in connective tissues,2) and proteoglycan sulfate, while decorin is moisture retention. In addition, because hyaluronan appears to play an important role in uterine cervical ripening and dilatation.

The hyaluronan levels of uterine cervix,7) cervical mucus,8) amniotic fluid,9) and serum10) also change during pregnancy. In addition, cervical ripening of rabbit is accelerated by the administration of hyaluronan to the uterine cervix.11) Collagenase activity is increased by the administration of hyaluronan to cell cultures of human myometrium in vitro,11) and cytokine production is promoted by adding hyaluronan to cultures of human uterine fibroblasts.12) On the other hand, uterine hyaluronan level and collagen degradation activity are increased by administration of PGE2 to the uterine cervix.13) Therefore, hyaluronan appears to play an important role in uterine cervical ripening and dilatation.

Generally, one of the physiological functions of hyaluronan is moisture retention. In addition, because hyaluronan synthase-2 knockout mouse cannot grow normally, it is thought that hyaluronan also plays an important role in organ maintenance.14,15) Recently it has been reported that the physiological functions of hyaluronan vary depending on its molecular weight.16)

It is generally known that cervical hyaluronan increases rapidly at delivery, and makes parturition easy. However, in previous reports, the molecular weight of hyaluronan in the uterine cervix has not been taken into consideration.

In this study, the molecular weight of hyaluronan in rabbit uterine cervix during late pregnancy was examined, and the uterine cervical ripening and dilatation activities of hyaluronan were investigated in vivo and in vitro.

MATERIALS AND METHODS

Animals Non-pregnant and pregnant Japanese white rabbits were used to study the effects of hyaluronan on the cervix. All animals were comparable in age (16 weeks) and body weight (2.5–3.5 kg). The present experiments were approved by the ethics committee of Shiseido Research Center in accordance with the guideline of the National Institute of Health.

Exogenous Hyaluronan Hyaluronan (Shiseido Co., Tokyo, Japan), with a mean molecular weight of 600—700 kilodaltons (kDa) (HA600—700), was used throughout the experiments. Hyaluronan of 990 kDa was treated at 85 °C for 24 h to obtain hyaluronan with a mean molecular weight of 620 kDa (600—700 kDa).17)

Preparation of Hyaluronan from Uterine Cervix and Estimation of Its Molecular Weight Uterine cervixes were removed from pregnant rabbits after euthanasia; fatty tissues were removed and the preparation was washed with a Ca2+- and Mg2+-free phosphate-buffered saline solution.
Amano Enzyme Inc., Nagoya, Japan) was added to 350 μl of 0.5% 2-cyanoacetoamide containing 7 M urea and 100 mM NaCl, and lastly eluted with an RF-550 spectrofluorometric detector (Shimadzu, Kyoto, Japan). Fluorescence of the eluted compounds was measured with an RF-550 spectrofluorometric detector (Shimadzu, Kyoto, Japan). Amano Enzyme Inc., Nagoya, Japan) was added to the filtrate and the mixture was incubated at 37 °C for 2 h. The fractionated hyaluronan was treated with two kinds of hyaluronidase, and the obtained unsaturated disaccharides was lyophilized against 50 mM Tris–HCl (pH 8.0) buffer containing 7 M urea, 10 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 10 mM N-ethylmaleimide. The flask was shaken at 4 °C for 16 h, and then dialyzed overnight against 50 mM Tris–HCl (pH 8.0) buffer containing 7 M urea, and 100 mM NaCl, and lastly eluted with a buffer containing 7 M urea and 500 mM NaCl. The eluted fraction was applied to a HiTrap Desalting Column (Amersham) and was desalted with 100 mM NH₄HCO₃. The desalted fraction was lyophilized.

The lyophilized samples were dissolved in a mobile phase containing 4 μl guanidium hydrochloride, 10 mM EDTA, 1 mM PMSF and 10 mM N-ethylmaleimide and applied to a double G6000PWxl column (Tosoh Co., Tokyo, Japan). The flow rate was 0.7 ml/min and fractions 1—50 were collected (1 fraction/min) over 10 min. Each fraction was applied to a HiTrap Desalting Column with a 10 μl acetate buffer (pH 6.0) containing 25 mM NaCl.

The fractionated hyaluronan was treated with two kinds of hyaluronidase, and the obtained unsaturated disaccharide was fluorescence-labeled. Streptomyces hyaluronidase (2TRU; Amano Enzyme Inc., Nagoya, Japan) was added to 350 μl of each fraction and incubated at 55 °C for 2 h. The digested fraction was then ultrafiltered through an Ultrafree C3GC system (molecular size cut-off 10000; Millipore Co., MA, U.S.A.). Hyaluronidase SD (15 μl; Seikagaku Kogyo Co., Tokyo, Japan) was added to the filtrate and the mixture was incubated at 37 °C for 2 h. The determination of unsaturated disaccharides was performed by fluorometric post-column HPLC as reported previously.18) Digested filtrate was mixed with the same volume of 50 mM sodium tetraborate buffer (pH 6.0) containing 0.5% 2-cyanoacetoamide, and heated at 100 °C for 60 min. Fluorescence of the mixture was detected with an RF-550 spectrofluorometric detector (Shimadzu, Kyoto, Japan). Fluorescence of the eluted compounds was monitored using ex=346 nm and em=410 nm. Molecular weight was calculated based on the elution times of standard hyaluronan samples of 988 kDa, 591 kDa, and 191 kDa (molecular weights were estimated from the intrinsic viscosity using the Mark-Houwink parameters reported by Laurent et al.19).

Estimation of Hyaluronan Levels in Serum Blood was drawn under anesthesia from the abdominal aortae of non-pregnant rabbits and pregnant rabbits at the 25th and 29th day of pregnancy. Blood was then centrifuged at 500×g for 30 min at 4 °C, and the serum was stored at −80 °C until analysis. The levels of hyaluronan in the sera were measured using a sandwich binding protein assay kit (Chugai Diagnostics Science Co., Ltd, Tokyo, Japan).

Effect of Hyaluronan Administration on Rabbit Uterine Cervix Rabbits (n=10) were prima gravida at the 23rd day of pregnancy. Five pregnant rabbits were aseptically given vaginal suppositories containing 20 mg of HA600—700 daily for three days. The other rabbits were used as controls and received the same amount of the vehicle (Pharmasol, Nof Co., Tokyo, Japan) used to prepare the suppositories. All the animals were euthanized on the 26th day of pregnancy after the third administration, and their uterine cervices were removed. All the cervices were fixed in 3% buffered formalin solution, and embedded in paraffin blocks. Cross-sections were cut 5—6 μm from the external os of the cervix (mid-cervix). Tissue sections were stained with Sirius red and fast green, which bind to collagen and noncollagenous proteins, respectively, to assess changes in collagen according to the method of Jimenez et al.20)

Tissue Culture of Rabbit Uterine Cervix Uterine cervixes were removed from pregnant rabbits at the 25th day of pregnancy under sterile conditions; fatty tissues were removed and the preparation was washed with PBS(−) containing 200 units/ml penicillin G and 100 μg/ml streptomycin. The finely minced tissue (100 mg) was cultured in 2 ml of Dulbecco’s modified Eagle’s medium (DMEM) with or without HA600—700 (final concentration: 1.0 mg/ml) at 37 °C in a CO₂ incubator under 5% CO₂–95% air as described previously.21) The culture medium was changed every 24 h and the harvested culture media were stored at −80 °C until determination of collagenolytic activity or PGE2. All experiments were conducted at least in triplicate and a typical set of data is shown.

Cell Culture of Rabbit Uterine Cervical Fibroblasts Uterine cervical fibroblasts were also prepared from pregnant rabbits at the 25th day of pregnancy and maintained in DMEM/10% (v/v) fetal bovine serum as described previously.22,23) In most experiments, cells up to the second passage were used. After confluence, the culture medium was changed to DMEM/0.2% (w/v) lactalbumin hydrolysate to estimate the production of collagenolytic activity, and HA600—700 (final concentrations: 0.01, 0.1 and 1.0 mg/ml) was added to the medium for 48 h. The harvested culture media were stored at −80 °C until use. All experiments were conducted at least in triplicate and a typical set of data is shown.

Measurement of Collagenolytic Activity The latent collagenase in the harvested culture medium of rabbit uterine cervical fibroblasts or tissue explants was activated by incubation with a final 1 mM 4-aminophenyl mercuric acetate (APMA) at 37 °C for 2 h. Collagenolytic activity was measured in terms of the degradation of fluorescent isothiocyanate (FITC)-labeled type I collagen using a type I collagenase assay kit (Yagai Co., Ltd., Yamagata, Japan), according to the manufacturer’s instructions. One unit of activity was defined as the amount that digests 1 μg of substrate per 1 min.

Measurement of PGE2 PGE2 in the culture medium was estimated using an enzyme immunoassay kit (EIA kit; Cayman Chemical Co., Ann Arbor, MI, U.S.A.) according to the manufacturer’s instructions.

Statistical Analysis The statistical significance of differences in hyaluronan levels was evaluated using Tukey’s test. The significance of differences in the tissue culture experiments was evaluated using a paired t-test, and that in the case of cell culture experiments was evaluated using Dunnett’s test.
RESULTS

Molecular Weight of Hyaluronan and Change of Hyaluronan Levels in Rabbit Uterine Cervices of Late Pregnancy

We investigated the molecular weight distribution of hyaluronan in rabbit uterine cervix on the 25th and 29th days of pregnancy. The main peak molecular weight was about 620 kDa in both tissues (Fig. 1). In addition, the hyaluronan peak at the 29th day was lower than that at the 25th day of pregnancy. However, a low-molecular-weight hyaluronan, less than 191 kDa, was also detected at the 29th day of pregnancy (Fig. 1). By contrast, hyaluronan was not detected in uterine cervix from non-pregnant animals.

Next, serum hyaluronan levels were compared. The serum hyaluronan level at the 25th day of pregnancy was 1.3 times that of non-pregnant rabbits. However, the difference was not statistically significant. At the 29th day of pregnancy serum hyaluronan was significantly increased to 2.3 times that of non-pregnant rabbits, and was also significantly increased to 1.7 times that on the 25th day of pregnancy (Fig. 2).

Hyaluronan Promoted Uterine Cervical Ripening in Rabbits

The molecular weight of the endogenous hyaluronan, which was increased in uterine cervixes of pregnant rabbits, was about 620 kDa. Therefore, hyaluronan in the molecular weight range of 600—700 kDa was considered to be similar to the endogenous hyaluronan, and was used to examine the influence of hyaluronan on uterine cervical ripening and dilatation. A vaginal suppository containing 20 mg of HA 600-700 was administered daily for three days to rabbits from the 23rd day of pregnancy. The uterine cervixes of the treated group became edematous, and exhibited detachment of collagen fiber bundles in comparison with the vehicle con-

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Fig. 1. Estimation of Molecular Weight of Hyaluronan in Rabbit Uterine Cervices

Rabbit uterine cervical tissues homogenized (10% (w/v)) in PBS(−) were centrifuged at 10000 × g for 20 min at 4 °C, and hyaluronan in the supernatant was purified as described in Materials and Methods. Molecular weights of standard hyaluronan were 988 (A), 591 (B), and 191 kDa (C).

Fig. 2. Changes of Serum Hyaluronan Levels in Pregnant Rabbits

Hyaluronan levels were estimated by sandwich binding protein assay. Values are the mean ± S.D. of five animals. ∗ and ∗∗, significantly different from the 29th day of pregnancy group (p < 0.05 and p < 0.01, respectively).

Fig. 3. Effects of Hyaluronan Administration on Collagen Fibrils in Uterine Cervix

Collagen fibrils in rabbit cervical connective tissues were stained with Sirius red/fast green. Panels (A) and (B), control cervix of pregnant rabbit. Collagen fibrils are seen as densely packed bundles. Panels (C) and (D), HA 600-700-treated cervix of pregnant rabbit. A marked decrease in collagen density and an increase in detachment of collagen fibrils can be observed. Magnification of (A) and (C) is ×40, and that of (B) and (D) is ×200.
trol group. These observations are regarded as indicating promotion of ripening (Fig. 3).

Effects of Hyaluronan on Collagenolytic Activity and PGE2 Production in Cultured Uterine Cervices  The effect of hyaluronan on other ripening indicators was investigated *in vitro*. Rabbit uterine cervical tissue at the 25th day of pregnancy was incubated with culture medium including HA600—700 1.0 mg/ml for 7 d. Figure 4 shows that the collagen degradation activity in culture medium was increased significantly by HA600—700 treatment in comparison with the control from the fourth culture day. Measurements were conducted after activation with APMA. 24) Since the collagenolytic activity was inhibited by EDTA and was unaffected by serine proteinase inhibitor, PMSF, it is suggested that this collagenolytic activity originated from collagenase (Table 1).

The effects of HA600—700 on PGE2 production were also investigated in cultured rabbit uterine cervix explants, and PGE2 levels in the culture media of the 4th day were estimated. PGE2 levels in the HA600—700-treated group increased significantly to 2.5 times the control (Fig. 5).

Furthermore, very similar effects of HA600—700 on the release of collagenolytic activity were observed in rabbit uterine cervical fibroblasts. When uterine cervical cells were treated with or without HA600—700 (0.1, 1.0 mg/ml) or IL-1β (positive control; 0.1 ng/ml) for 48 h, IL-1β significantly enhanced the release of collagenolytic activity as compared to the non-treated control; 1.24±0.06 vs. 0.63±0.02 (units/ml) (*p<0.001). HA600—700 (0.1, 1.0 mg/ml) significantly augmented the release of collagenolytic activity in a dose-dependent manner as compared with control; 0.87±0.03 (*p<0.001) and 1.05±0.04 (units/ml) (p<0.001), respectively (data not shown).

**DISCUSSION**

Hyaluronan has a linear structure with a range of molecular weights and is widely distributed in organisms. Most hyaluronan has a role in moisture retention in tissues, because hyaluronan has a high affinity for water.25) The hyaluronan levels of the uterine cervix,7 cervical mucus,8 amniotic fluid,9 and serum10 vary during gestation. Here, we focused on the function of hyaluronan in the uterine cervix.

First, the molecular weight of purified hyaluronan from rabbit uterine cervixes of non-pregnant rabbits and animals at the 25th and 29th days of pregnancy was measured using gel-permeation chromatography. The molecular weight of hyaluronan in uterine cervix was predominantly about 620 kDa. This is small in comparison with the hyaluronan of more than 1000 kDa in normal rabbit synovial fluid and skin.20) A small amount of low-molecular-weight hyaluronan, less than 191 kDa, was also detected in uterine cervix at the 29th day of pregnancy.

Hyaluronan was undetectable in non-pregnant uterine cervix, but was detected in uterine cervix at the 25th day, then was decreased at the 29th day of pregnancy. Rath *et al.*26) reported that a remarkable increase of hyaluronan in cervix occurs in the first stage of dilation, and hyaluronan sharply decreases with the onset of regular labor. Our results are in good agreement with this. It was morphologically observed that hyaluronan of 620 kDa promotes cervical ripening. Moreover, in an *in vitro* study, this hyaluronan increased total collagen degradation activity, which is one of the indicators of ripening.

In contrast, hyaluronan level in serum at the 29th day of pregnancy was increased in comparison with that at the 25th day. This suggests that the increase in serum hyaluronan is derived from the cervix because sequential change of hyaluronan in human cervix25) is similar to human serum.10)
Obara et al. reported that hyaluronidase activity increases with time from the second trimester of pregnancy to parturition, in human uterine cervical mucus. Therefore, an increase of hyaluronan level in serum and a decrease of hyaluronan level in uterine cervix at the 29th day of pregnancy can be explained by the intervention of hyaluronidase; i.e., hyaluronan of uterine cervix is degraded by hyaluronidase and hyaluronan is transferred from the ripened uterine cervix to serum during late pregnancy.

On the other hand, it is well-known that hyaluronan has been detected in immune-related diseases and inflammatory sites and it induces various kinds of inflammatory factors. Therefore, it may regulate both immune and inflammatory reactions. Kobayashi et al. reported that in uterine fibroblasts, hyaluronan (800 kDa) enhances cytokine production, though hyaluronan of 10—100 kDa does not. Therefore, we speculate that 620 kDa endogenous hyaluronan promotes uterine cervical ripening and dilatation, but less than 191 kDa hyaluronan does not. As regards hyaluronan synthesis, it is reported that HAS is induced by PGE2, IL-1, EGF, and interferon-γ in various cells.

In human amniotic fluid and cervicovaginal fluid, IL-1 production increases in term pregnancy. The PGE2 level in amniotic fluid also increases at term and hyaluronan regulates PGE2 production in amnion cells. These facts indicate that hyaluronan regulates cervical ripening and dilatation through cytokines and PG. We therefore investigated whether hyaluronan regulates PGE2 production in pregnant uterine cervix. In tissue culture, PGE2 production was increased significantly by hyaluronan treatment, but in cell culture, its production was not changed significantly (data not shown).

This result suggests that the increase in PGE2 in tissue culture was due to production from endothelial cells and/or leukocytes, or was a secondary product derived from tissues stimulated with endogenous cytokines such as IL-1 and tumor necrosis factor-α. Hyaluronan is an inducer of IL-1 production by human monocytes and rabbit macrophages.

On the other hand, PGE2 administered to pregnant uterine cervix increased hyaluronan and collagenase. Furthermore, PGE2 production and COX-2 expression are regulated in rabbit uterine cervical cells by cytokines and sex hormones. Therefore, we suggest that hyaluronan, cytokines and PGs, and other mediators in the uterine cervix independently cross-talk, and thereby promote cervical ripening and dilatation for delivery.

In conclusion, our results indicate that hyaluronan of about 620 kDa, produced in the uterine cervix before delivery, augments production of collagenase and PGE2, thereby promoting cervical ripening and dilatation. Therefore, hyaluronan in the uterine cervix not only maintains the water content, but also acts as a cytokine-like factor. We are currently investigating the mechanisms of hyaluronan production in uterine cervix.

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