Cyclic mechanical stretch augments hyaluronan production in cultured human uterine cervical fibroblast cells

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Hyaluronan (HA) a glycosaminoglycan with high affinity for water molecules stimulates local inflammatory reactions. Parturi- tion causes a dramatic increase in the amount of HA fragments in the uterine cervix, thereby contributing to a rapid softening as well as opening of the cervical canal, i.e. cervical ripening. The aim of this study was to investigate the possible involvement of cyclic distension caused by labour in the augmentation of HA production during cervical ripening. Immunohistochemistry and/or RT–PCR detected hyaluronan synthase (HAS)1, 2 and 3 in samples of human cervical tissue obtained from pregnant women. Labour-like cyclic mechanical stretch for 24, 36 and 48 h significantly enhanced the secretion of HA, from cultured human uterine cervical fibroblast (C) cells, 128.7, 151.4 and 173.2%, respectively, concomitant with a significant augmentation of HAS1 (36, 48 h), HAS2 (24, 36 and 48 h) and HAS3 (48 h) mRNA expression. Cyclic mechanical stretch for 12, 36 and 48 h increased molecular size of the HA secreted from C cells. In conclusion, cyclic mechanical stretch of the uterine cervix caused by the presenting part of the fetus in labour may contribute to the increase in the secretion of HA during the process of cervical ripening.

Key words: cervical ripening/hyaluronan synthase/hyaluronan/mechanical stretch/uterine cervix

Introduction

During pregnancy the uterine cervical canal remains tightly closed and retains the fetus in the uterine cavity which accommodates it until the onset of active labour, when a rapid softening and dilatation of the uterine cervix, i.e. cervical ripening. This is concomitant with vigorous cyclic contractions of the uterine corpus, followed by the delivery of the neonate (Cunningham et al., 2005).

Uterine cervical ripening is characterized by two dramatic phenomena, namely intensive local leukocyte infiltration and the rapid remodeling of extracellular matrix proteins, such as collagen, proteoglycan and structural glycoprotein (Danforth et al., 1974; Kleissl et al., 1978; Ito et al., 1979; Junqueira et al., 1980; Liggins, 1981; Uldbjerg et al., 1983; Kelly, 1994). Among the complicated changes of extracellular matrix proteins during cervical ripening, a local increase of hyaluronan (HA) in the uterine cervix, especially HA fragments, is hypothesized to play a crucial role (Golichowski et al., 1990; Cabrol et al., 1990, 1991; Osmers et al., 1993; Rath et al., 1993; El Maradny et al., 1997; Kobayashi and Terao, 1997; Ogawa et al., 1998; Belayet et al., 1999; Ludmir and Sehdev, 2000; Kavanagh et al., 2001; Obara et al., 2001) because of the high affinity for water and collagenolytic as well as inflammatory effects of these fragments (McKee et al., 1996, 1997; Kobayashi et al., 1998; Lee and Spicer, 2000; Li et al., 2000; Haslinger et al., 2001; Sun et al., 2001). It has been reported that parturition significantly increases the concentration of HA in plasma (Kobayashi et al., 1999) as well as the local concentration of HA in the uterine cervix (von Maillot et al., 1979; Osmers et al., 1993). An accumulation of HA in the extracellular matrix results in the softening and swelling of the uterine cervix because of its unique elastic properties associated with its high affinity for water (Rajabi et al., 1992). In addition to its function as a structural protein in the extracellular matrix, HA triggers various biological events such as inflammation, proliferation, angiogenesis, invasion, transformation and migration (McDonald and Camenisch, 2002; Hascal et al., 2004; Spicer and Tien, 2004). It was previously reported that HA stimulates the secretion of interleukin-8 (IL-8), IL-1 and tumour necrosis factor alpha (TNF-α) (El Maradny et al., 1997; Kobayashi and Terao, 1997). These inflammatory cytokines play important roles in the acceleration of cervical ripening (Yoshida et al., 2001; Cunningham et al., 2005). HA fragments are reported to have strong ability to induce inflammatory reactions, compared to high molecular weight HA (McKee et al., 1996, 1997; Haslinger et al., 2001). Thus, both increase and fragmentation of HA play pivotal roles in the dramatic remodeling of the extracellular matrix in the process of cervical ripening.

Cervical ripening usually proceeds in parallel with the progression of labour, i.e. the cyclic contractions of the uterine corpus (Cunningham et al., 2005). Braxton-Hicks uterine contractions as well as active labour cause a cyclic descent of the presenting part of the fetus, applying cyclic stretch to the uterine cervix in parturition (Cunningham et al., 2005). We recently developed culture models of cyclic stretch of the myometrium (Korita et al., 2002, 2004), fetal membrane (Terakawa et al., 2002) and uterine cervix (Yoshida et al., 2002; Takemura et al., 2004) by using a computer-operated vacuum-driven cyclic stretch system,
which mimics, at least partly, the characteristic stimulation from cyclic distension of the uterine cervix during parturition. Using this in vitro model, we have found that a physical force, labour-like cyclic mechanical stretch, augments production of matrix metalloproteinase-1 (MMP-1) (Yoshida et al., 2002) and inflammation associated chemokines, IL-8 and monocyte chemotactic protein-3 (MCP-3) (Takemura et al., 2004), in cultured human uterine cervical fibroblast (CxF) cells prepared from pregnant women. These findings suggest a novel role for cyclic mechanical stretch caused by labour, as an initiator of various chemical reactions expediting cervical ripening and followed by delivery of the conceptus, in addition to the stimulation with inflammation-associated bioactive substances, such as nitric oxide, inflammatory cytokines, prostaglandin F2α, and so on (Yoshida et al., 2001, 2002). The aim of this study was to investigate the possible involvement of cyclic mechanical stretch in the dramatic augmentation of the production and/or fragmentation of HA in human uterine cervix during the process of cervical ripening. HA is synthesized by hyaluronan synthase (HAS)1, HAS2 and HAS3 (Itano and Kimata, 2002). HAS1 and HAS2 synthesize high molecular weight HA, whereas HAS3 synthesizes low molecular HA (Itano et al., 1999). We investigated (i) whether HAS1, HAS2 and HAS3 are expressed in human cervix, (ii) whether labour-like cyclic mechanical stretch augments secretion of HA from CxF cells, (iii) whether labour-like cyclic mechanical stretch alters the molecular size of the HA secreted from CxF cells and (iv) whether labour-like cyclic mechanical stretch enhances HAS1, HAS2 and HAS3 mRNA expression in CxF cells.

Materials and methods

Reagents
All reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and were of analytical grade unless otherwise indicated.

Collection of cervical tissues
Uterine cervical tissue samples were obtained after hysterectomy with informed consent from two premenopausal non-pregnant women, two first trimester pregnant women (9 and 11 weeks of gestation), one second trimester pregnant woman (20 weeks of gestation) and two term pregnant women before onset of labour (37 and 38 weeks of gestation). The tissues were collected approximately 4–7 mm depth from endocervical side, after mechanically removing cervical epithelium. Hysterectomy was carried out for gynecological diseases, such as uterine myoma (9 and 11 weeks of gestation), uterine cervical intraepithelial neoplasia (37 and 38 weeks of gestation) or ovarian cancer (20 weeks of gestation). The tissues from two pregnant women at term were used to establish cultured human uterine CxF cells. An ovary was obtained from a premenopausal non-pregnant woman and used as a positive control for HASs expression. Samples were either snap frozen in liquid nitrogen in blocks for mRNA extraction or embedded in optical cutting temperature (OCT) compound (Sakura Finetek Inc., Torrance, CA, USA) for immunohistochemical examination and stored at −80°C until used. In cases of uterine cervical intraepithelial neoplasia and ovarian cancer, histological examination of tissues close to the specimen showed no neoplastic cells. The study was approved by the ethics committee on human research at Kyoto University Graduate School of Medicine.

Immunohistochemical detection of HAS1 and HAS2
Six-micrometer-thick sections were incubated for 1 h at room temperature with rabbit polyclonal peptide antiserum anti-HAS1 (1 μg/ml) or anti-HAS2 (1 μg/ml) kindly donated by Professor Paraskevi Heldin (Jacobson et al., 2000). Normal rabbit serum (Dako Co., Carpinteria, CA, USA) was used as a negative control. Staining was detected using an avidin-biotin-peroxidase method kit (ELITE ABC, Vector Laboratories, Burlingame, CA, USA) with 3,3′-diaminobenzidine as previously described (Itoh et al., 1998).

RT–PCR analysis of HAS1, HAS2 and HAS3
Total RNA was extracted from cervical tissue and CxF cells as previously described (Masuzaki et al., 1997). After the reverse transcription of 2 μg of total RNA from human pregnant and non-pregnant uterine cervixes and CxF cells using oligo(dT) primer (Promega, Madison, WI, USA) and SUPERSCRIPT™II (GibcoBrl, Rockville, MD, USA), the resulting single-stranded cDNA was subjected to PCR. Forward and reverse primers used for amplifying portions of the cDNAs of human HAS1, HAS2 and HAS3 are summarized in Table I. Forward and reverse primers for the human GAPDH coding region were purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA).

Preparation of cultured human uterine CxF cells and experimental protocol for cell culture and stretch experiments
CxF cells were prepared by the explant method as previously reported (Yoshida et al., 2001, 2002; Takemura et al., 2004). An immunofluorescence study showed 99% positive staining for vimentin in CxF cells at the sixth passage and less than 1% positive staining for both cytokeratin and α-smooth

Table I. Forward/reverse primers used in the RT–PCR analysis and forward/reverse primers and Fam/Tamra probes used in the quantitative–PCR analysis for amplifying portions of the cDNAs of human HAS1 (GeneBank Accession No. BC035837; Strausberg et al., 2002), HAS2 (U54804; Watanabe et al., 1996) and HAS3 (AF232772; Spicer et al., 2000)

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HAS, hyaluronan synthase.
carried out in quadruplicate wells. Observations were expressed widely in cervical tissues. The positive staining in glandular cells was prominent as compared with that in stromal cells. An apparently strong immunostaining of both HAS1 and HAS2 was observed in cervical tissue obtained in the third trimester of pregnancy, indicating that both HAS1 and HAS2 were expressed in these tissues.

Measurement of HA concentration and molecular weight in the culture medium of CxF cells and human skin fibroblast cells

HA levels in the culture medium were measured using a commercially available ELISA kit, which can detect HA with molecular weights greater than 100 kDa (Corgenix Medical Co., Westminster, CO, USA). The molecular weight of HA was estimated by gel filtration (TSK SUPER AW6000, Tosoh Co., Tokyo, Japan), as previously described (Fujita et al., 2002). The specific HA area was confirmed by enzymatic digestion with hyaluronidase Streptomyces (Seikagaku Co., Tokyo, Japan) (Fujita et al., 2002).

Quantitative RT–PCR analysis of HAS1, HAS2 and HAS3

The mRNA expression of human HAS1, HAS2 and HAS3 in the CxF cells, with or without cyclic stretch, was measured by real-time quantitative RT–PCR using Taqman™ technology (Model 7700 sequence detector, Applied Biosystems, Foster City, CA, USA) (Yoshida et al., 2001). The forward and reverse primers, and Fam/Tamra probes used for the targeted amplification of part of the cDNAs of human HAS1, HAS2 and HAS3 are summarized in Table I. The forward and reverse primers, and Joe/Tamra probes used for the human GAPDH coding region were purchased from Applied Biosystems. The mRNA expression was estimated by dividing the threshold cycle (CT) values by GAPDH CT values as previously described (Yoshida et al., 2001). Four independent experiments were carried out in quadruplicate wells.

Statistical analysis

Values were expressed as the mean ± SEM. Statistical significance was assessed with the Mann–Whitney U test and analysis of variance (ANOVA) followed by Fisher’s protected least significance difference test when applicable. P-values less than 0.05 were regarded as significant.

Results

The expression of HAS1, HAS2 and HAS3 in non-pregnant and pregnant uterine cervical tissues and cultured CxF cells

RT–PCR analysis revealed HAS1, HAS2 and HAS3 mRNA expression in the non-pregnant, first and third trimester pregnant uterine cervix samples and cultured CxF cells (Figure 1). Immunohistochemistry showed strong positive staining for HAS1 and HAS2 in both stromal cells and glandular cells in the pregnant uterine cervix at the second and the third trimesters of pregnancy (Figure 2), indicating that both HAS1 and HAS2 were expressed widely in cervical tissues. The positive staining in glandular cells was prominent as compared with that in stromal cells. An apparently strong immunostaining of both HAS1 and HAS2 was observed in cervical tissue obtained in the third trimester of pregnancy, compared to that in the second trimester or in a non-pregnant woman.

Figure 1. RT–PCR analysis of the mRNA expression of hyaluronan synthase (HAS1, HAS2 and HAS3). Total RNA was obtained from uterine cervix of a premenopausal non-pregnant woman (NP), first trimester pregnant women at 11 weeks of gestation (1st), second trimester pregnant woman at 20 weeks of gestation (2nd) and third trimester pregnant woman at 37 weeks of gestation (3rd), cultured human uterine cervical fibroblast (CxF) cells and ovarian tissue as positive control. Date are representative results from individual specimens.

Negative controls, using normal rabbit serum, showed greatly reduced staining (Figure 2). The ovary was used as a positive control (data not shown). During the culture study of CxF cells, the basal secretion of HA was significantly and time-dependently increased (Figure 3) concomitant with HAS1 and HAS3, but not HAS2, mRNA expression (Figure 4).

The effect of cyclic mechanical stretch on the secretion of HA from CxF cells

The HA concentrations in the medium of CxF cells after 24, 36 and 48 h of incubation under stimulation with cyclic mechanical stretch were significantly higher than those in the medium of cells without stimulation (n = 3 and P < 0.01 for all comparisons) (Figure 3). On the other hand, the HA concentration in the medium of CxF cells after 12 h of incubation with cyclic mechanical stretch was similar to that without stretch (Figure 3).

The molecular weights of HA in the culture medium after cyclic mechanical stretch for 12, 36 and 48 h were larger than those without stimulation, respectively, although no such difference was observed after 24 h of stimulation (Table II).

The effect of cyclic mechanical stretch on the secretion of HA from cultured human skin fibroblast cells

Cyclic mechanical stretch for 12, 24, 36, and 48 h also significantly increased the secretion of HA from cultured human skin fibroblast cells (Table III). The HA molecular weight in the culture medium after cyclic mechanical stretch for 48 h was 1350 kDa, larger than that without the stimulation, 1043 kDa. We could not assess the molecular weights of HA in the culture medium after cyclic mechanical stretch for 12, 24 and 36 h because of low HA concentration in the culture medium.

Discussion

This study revealed that the mRNA of all three HASs was expressed in human uterine cervix from pregnant women (Figure 1). Both
HAS1 and HAS2 were detected by immunohistochemistry in stromal cells as well as glandular cells (Figure 2). The apparent immunostaining in glandular cells suggested a possible contribution to cervical ripening. It is a future aim to clarify whether HA secretion from glandular cells affects the extracellular matrix of stromal cells. In this study we investigated the possible effect of cyclic mechanical stretch on the secretion of HA and the mRNA expression of HASs in C×F cells by using an in vitro culture model of labour-like cyclic mechanical stretch (Korita et al., 2002, 2004; Terakawa et al., 2002; Yoshida et al., 2002; Takemura et al., 2004). HA secretion from C×F cells was significantly elevated after 24–48 h stimulation of labour-like cyclic mechanical stretch, simultaneously with a significant increase of HAS2 mRNA expression. However, significant augmentation of HAS1 and HAS3 mRNA expression was only observed after 36 and 48 h and 48 h stimulation, respectively. Therefore it is suggested that labour-like cyclic mechanical stretch may increase enzyme activities of HASs before the elevation of their mRNA expression. Further enzymatic analysis is necessary to prove such speculation. Therefore, it is plausible that cyclic mechanical stretch of the uterine cervix during labour increases local synthesis of HA and promotes remodelling of the extracellular matrix, contributing to the initiation and/or acceleration of cervical ripening, since cyclic descent of the fetus through contraction of the uterine corpus is a characteristic feature of labour (Cunningham et al., 2005).

Cyclic mechanical stretch also augmented the secretion of HA from cultured human skin fibroblast cells (Table III). However, HA secretion was greater from C×F cells than human skin fibroblast cells (Figure 3 and Table III), which is in agreement with a previous report of another group (Tanaka et al., 1994), suggesting that uterine C×F cells have distinct characteristics concerning HA production, compared to skin fibroblast cells. Cyclic stretch was also reported to increase production of HA in bovine chondrocytes (Yamazaki et al., 2003) as well as in rat lung (Mascarenhas et al., 2004).

Figure 2. Immunohistochemical staining for hyaluronan synthase (HAS)1 and HAS2 in the uterine cervix from a premenopausal non-pregnant woman (NP), second trimester pregnant women at 20 weeks of gestation (2nd), third trimester pregnant women at 37 weeks of gestation (3rd) and negative control of the same second trimester specimen using normal rabbit serum (Neg).

HAS1 and HAS2 were detected by immunohistochemistry in stromal cells as well as glandular cells (Figure 2). The apparent
Effect of cyclic mechanical stretch on the molecular weight of hyaluronan (HA) in the culture medium of CxF cells (Table II), suggesting that cyclic mechanical stretch may not be directly associated with the fragmentation of HA during cervical ripening. HAS1 and HAS2 synthesize high molecular weight HA, whereas HAS3 synthesizes low molecular weight HA (Itano et al., 1999). Cyclic mechanical stretch significantly augmented the mRNA expression of HAS1, HAS2 and HAS3 in CxF cells (Figure 4). However, cyclic mechanical stretch for 12, 36 and 48 h, but not 24 h, increased the molecular weight of HA in the culture medium of C x F cells (Table III), suggesting that the contribution of HAS3 to the production of HA in Cx F cells is not as prominent as that of HAS1 and/or HAS2. More detailed analyses of the protein expression as well as enzymatic activity of both HASs and degradative enzymes of HA are necessary to investigate the mechanism behind the rather stable molecular weight of HA following cyclic mechanical stretch for 24 h, compared to that after 12, 36 and 48 h of stimulation (Table III). A pilot study in our laboratory revealed mRNA expression of HA degradative enzymes, such as hyaluronidase1, hyaluronidase2 and sperm adhesion molecule 1, in cervical tissues from pregnant women as well as C x F cells (Takemura M and Itoh H, unpublished findings). Because inflammatory cytokines, such as IL-1β and TNF-α, are reported to increase hyaluronidase1 mRNA expression in human periodontal ligament fibroblast cells (Ohno et al., 2002) and inflammatory reactive oxygen species were reported to degrade HA (Moseley et al., 1997), it would be interesting to investigate a possible contribution of inflammatory reactions to the fragmentation of HA in the process of cervical ripening.

Caution is needed when using present in vitro findings to explain the physiological phenomena caused by cyclic stretch during the complicated process of cervical ripening. In this study, significantly elevated HA section was evident after 24 h of stimulation, whereas most cases of normal deliveries are completed within 24 h. At present we have no clear explanation for this discrepancy. Nevertheless the present in vitro data strongly suggests that cyclic mechanical stretch during labour stimulates the production of HA, especially the larger form. It was reported that progesterone inhibited HA secretion from uterine cervical fibroblast cells (Tanaka et al., 1994; Straach et al., 2005; Uchiyama et al., 2000), suggesting a possible involvement of decrease of progesterone and/or progesterone receptors in the regulation of HA synthesis in parturition. Therefore, steroid hormones, together with cyclic stretch by labour, may cooperate to augment HA production in cervical ripening. The HA may help to promote the softening and swelling of the extracellular matrix, as a structural protein, as well as the inflammatory reactions, as a bioactive substance, in the cascade of events that occur during cervical ripening. HA causes such bioactive effects through its receptor CD44 (Fitzgerald et al., 2000), which was found to be widely expressed in human uterine cervix stromal cells in our preliminary study using immunohistochemistry (Takemura M and Itoh H, unpublished findings). In the present in vitro study, basal secretion of HA increased during the culture study (Figure 3), concomitant with

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Values are expressed as means and SEM of HA concentration in the culture medium from quadruplicate wells.

* P < 0.05 versus 12 h without stretching.
† P < 0.001 versus no stretching.
‡ P < 0.005 versus 12 h with stretching.
HAS1 and HAS3 mRNA expression, but not with HAS2 mRNA expression (Figure 4), suggesting a possible association of other unknown factors with the regulation of HA production. Because this study did not provide the direct evidence that HA from CxF cells induces local inflammatory effects, further investigation will be needed to elucidate the contribution of HA to cervical ripening.

In summary, HA is produced by CxF cells in a manner that can be regulated at least partly by a labour-like cervical mechanical stretch. Based on these findings, we suggest that cyclic stretch in labour plays an important role in the augmentation of HA production during the process of cervical ripening in humans.

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Stretch and hyaluronan production in uterine cervix


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