Dehydroepiandrosterone sulphate promotes hyaluronic acid-induced cervical ripening in rabbits

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Introduction

Ripening of the uterine cervix at the end of gestation is an essential step of normal parturition. The uterine cervix consists mainly of connective tissue composed of collagen, proteoglycan and structural glycoprotein. Marked changes in these connective tissue components occur during dilatation and ripening of the cervix at term (Danforth et al., 1974; Karube et al., 1975; Kleissl et al., 1978; Ito et al., 1979; Kitamura et al., 1980; Uldbjerg et al., 1983). However, the hormonal regulation of these changes in connective tissue metabolism has not yet been elucidated.

Dehydroepiandrosterone 3-sulphate (DHEA-S) is an androgen which is produced by the adrenal gland (Baulieu et al., 1965) and is converted to oestrogen in human placentae (Siiteri and MacDonald, 1966). Increases in the concentration of DHEA-S in baboon serum (Townsley and Pepe, 1977), human umbilical cord plasma (Parker et al., 1982) and human uterine cuffses (Tahara et al., 1985) were found to parallel the progress of pregnancy, indicating that DHEA-S is concerned directly or indirectly with uterine cervical dilatation in late pregnancy. It has been reported that pharmacological doses of DHEA-S induced increases in the Bishop score and in the release of collagenase from human cervical explants (Mochizuki and Tojo, 1980). Our previous observation demonstrates that vaginal application of DHEA-S into rabbits promotes interleukin (IL) 8-induced cervical ripening (Maradny et al., 1996). Recently, we reported that DHEA-S induces the production of IL-8 and the expression of IL-8 receptors in cervical fibroblasts (Kanayama et al., 1998), suggesting that DHEA-S regulates the autocrine system of IL-8 through the expression of IL-8 receptors. Evidence has also been presented that DHEA-S enhances hyaluronic acid (HA) production and also acts as a stimulator of HA synthesis in human cervical fibroblasts (Tanaka et al., 1997).

HA is one of the primary constituents of the extracellular matrix of the human uterine cervix. It has been reported that the amount of HA increases markedly at the last stage of pregnancy and decreases rapidly after parturition (Golichowski et al., 1980; Shimizu et al., 1980; Osmers et al., 1993). These dramatic changes suggest that HA plays an important role in the regulation of cervix function during parturition. An increased HA concentration in cervical connective tissue has been described previously as one of the biological features of cervical ripening (Osmers et al., 1993). A previous report suggests that HA stimulates collagenase and elastase activity in cervical tissue culture (Hiro et al., 1986). In non-pregnant and pregnant rabbits treated with vaginal suppositories containing HA, pronounced ripening and dilatation of the uterine cervix have been found, together with a decreased collagen concentration and increased collagenase and gelatinase activities (Maradny et al., 1997). It has also been reported that HA stimulates IL-8 production and expression (Noble et al., 1993; McKee et al., 1996; Kobayashi and Terao, 1997; Maradny et al., 1997).

DHEA-S plays a role in the effect of IL-8 and its receptors...
in the cervix, and the production of IL-8 in HA-stimulated fibroblasts implies the role of an autocrine stimulatory loop in ripening of the uterine cervix. Based on these reports, it seemed important to investigate the effect of DHEA-S on the HA-induced cervical ripening process; consequently, our studies in rabbits were initiated.

Materials and methods

These studies were carried out in 25 New Zealand White rabbits on day 23 of pregnancy. All rabbits were primigravida and comparable in terms of bodyweight and age. The animals were treated either with placebo or drugs in the form of vaginal suppositories, once daily for 3 days. Vaginal suppositories were prepared with 500 µl Witepsol-50 bases (WT-50; Adeps solidus: Mitsuha Co., Tokyo, Japan), all products of cocoa butter of plant origin. The prepared suppositories were conical-shaped and melted at 37°C. The suppositories using Witepsol bases were quick to melt and thus attached poorly to tissues. Drug release from the suppositories (measured by the recommended method) occurred over a short time period. Animal experimentation described in this manuscript was approved by the Research Committee of Laboratory Animals, Hamamatsu University School of Medicine.

The rabbits were allocated at random to five equal groups and treated with suppositories containing 1 mg HA (Kanebo Co., Tokyo, Japan) (group I) or 30 mg DHEA-S (Oncogene Science Inc., MA, USA) (group II). Additional rabbits received suppositories containing either 30 mg DHEA-S + 0.1 mg HA (group III) or 30 mg DHEA-S + 1 mg HA (group IV). Control rabbits (group V) were treated with 500 µl Witepsol-50 bases.

The animals were killed at 24 h after the last suppository dose. The reproductive tract was immediately located and the cervix excised from all animals. One cervix section from each animal was used to measure water content, followed by the measurement of collagenase, elastase and gelatinase activities; the other cervix section was fixed in 10% buffered formalin for 24 h. Following paraffin embedding, blocks were serially sectioned at a thickness of 5 µm using a Reichert Jung 2050 motorized automatic microtome. The sections were stained with either haematoxylin followed by a 1% eosin counterstain (H&E), or stained with picrosirius red (Sirius red F3BA; Chroma-Gesellschaft, Germany). The relative collagen concentration was assessed histologically after staining with picrosirius red (Sirius red F3BA; Chroma-Gesellschaft, Germany) (group I) or 30 mg DHEA-S (Oncogene Science Inc., MA, USA) (group II). Additional rabbits received suppositories containing either 30 mg DHEA-S + 0.1 mg HA (group III) or 30 mg DHEA-S + 1 mg HA (group IV). Control rabbits (group V) were treated with 500 µl Witepsol-50 bases.

The water content of each cervix was measured using an IM-3SCV device (Fuji Technica Co., Osaka, Japan). This is an infra-red spectrophotometric technique which compares absorbance at a wavelength of 1.45 nm with absorbance at reference wavelengths of 1.3 µm and 1.6 µm (Sumimoto and Terao, 1993). Five different points on the cervix were measured and the mean was calculated.

Measurement of collagenase, elastase and gelatinase activities

Each cervix was homogenized in 1 ml ice-cold phosphate-buffered solution (PBS, pH 7.6). After two cycles of freeze–thaw extraction, the sample was sonicated (30 W, 120 pulses, and 30% duty, W-220 type; Heat Systems Ultrasoonics, New York, NY, USA) and then centrifuged at 10,000 g for 20 min at 4°C. The supernatant was used to measure collagenase, elastase and gelatinase activities as described previously (Maradny et al., 1997). Collagenase activity in the sample was estimated using a highly specific kit (collagenase type 1 activity measurement: Yagai Co., Cosmo-Bio, Tokyo, Japan), whereas elastase activity was determined by a specific chromogenic substrate for granulocyte elastase S-2484 (l-pyroglutamyl-l-prolyl-l-valine-p-nitranilide; KABI Diagnostic, Molndal, Sweden). Gelatinase activity was measured using a specialized kit (Yagai Co., Cosmo-Bio, Tokyo, Japan). For each enzyme, 1 unit of activity was defined as the quantity of enzyme that digested mg of substrate in 1 min.

Immunohistochemistry of the cervix

Paraffin-embedded tissue was deparaffinized in xylene baths, rehydrated through graded 95% alcohol, and finally rinsed in PBS at pH 7.2. Endogenous peroxidase was blocked by fixation in 3% hydrogen peroxide in methanol for 20 min at 23°C. Bovine serum albumin–phosphate-buffered solution (BSA–PBS, 2%) was applied to the slides for 1 h. To identify neutrophils, anti-rabbit RT2 monoclonal antibody was used (Cedarlane Laboratories Limited, Hornby, Canada). RT2 (1:100) was added to the sections, which were kept overnight at 4°C, followed by PBS rinsing (Ponsard et al., 1986). The secondary antibody (goat anti-rabbit; DAKO, CA, USA) was applied for 2 h at room temperature, followed by PBS rinsing. Avidin–biotin–peroxidase complex (DAKO) was added for 1 h, followed by PBS washing. The sections were counterstained in haematoxylin and dehydrated, then examined under light microscopy at ×200. Negative controls received the same treatment, though non-immune mouse serum was used instead of primary antibody. For a positive control, neutrophils in the tissue were used. The number of neutrophils was estimated by counting the number of stained extravascular cells within a lined grid (10×10 squares) occupying an area on the section of 0.125 mm² using a ×20 objective and ×10 eyepiece. Neutrophils in cervical connective tissue were counted in one cervix from each animal (between five and seven randomly chosen areas) and the mean was calculated.

Assay of relative collagen concentration

The relative collagen concentration was assessed histologically after staining with picrosirius red (Sirius red F3BA; Chroma-Gesellschaft Schmild GmbH, Kongen, Germany) as described previously, and validated as a histological method to determine the polymerized collagen concentration of tissues, including the cervix (Junqueira et al., 1979). The histological analysis was performed by measuring the optical density (percentage polarized light transmission) from five random fields of the connective tissue of each biopsy, and the mean optical density calculated. An image analyser was employed for all histological measurements (microscope; Olympus IMT-2; videocamera; SIT C2400-80 and computer analyser system with ARGUS-100; Hamamatsu Photonic, Hamamatsu, Japan). The principle of picrosirius red staining is that, the greater the collagen concentration, the greater the birefringence, and hence the greater the percentage of light transmission.

Statistical analysis

Data are expressed as mean ± SD of all experiments. Analysis of variance (ANOVA) for factorial measurement followed by Scheffe’s F analysis was used for multiple comparisons. A probability of < 0.05 was considered to be significant for all comparisons.

Results

Number of neutrophils

The number of neutrophils was significantly increased in the cervical connective tissue of rabbits treated with HA, DHEA-S, DHEA-S + 0.1 mg HA, DHEA-S + 1 mg HA (P < 0.03, P < 0.05, P < 0.009, P < 0.0001 respectively), compared with control (Figure 1). Treatment with DHEAS + 1 mg HA showed a remarkable increase in neutrophil infiltration (P < 0.001) over the DHEA-S + 0.1 mg HA approach.
Relative collagen concentrations
As shown in Figure 2, the relative collagen concentration was significantly decreased in HA, DHEA-S, DHEA-S + 0.1 mg HA, DHEA-S + 1 mg HA (P < 0.009, P < 0.01, P < 0.0007, P < 0.0001 respectively), compared with control. The collagen concentration in rabbits treated with DHEA-S + 1 mg HA was markedly decreased (P < 0.002) compared with DHEA-S + 0.1 mg HA treatment.

Morphology with H&E staining
Treatment with DHEA-S + 1 mg HA revealed increased vascularity (Figure 3c), with massive dilatation of blood vessels. The density of the collagenous network markedly decreased and became loose, whereas the density of ground substances increased. Histological changes of a lesser degree were observed in cervices treated with HA (Figure 3a) and DHEA-S (Figure 3b). Control cervix (Figure 3d) showed a dense and firmly closed cervical ring, with collagenous networks and smooth muscle layers lying close together, the connective tissues compact, and the blood vessels small and non-dilated.

The changes in cervical water content and collagenase, gelatinase and elastase activities after suppository treatment are summarized in Table I. The water content was significantly increased in cervices treated with HA, DHEA-S, DHEA-S + 0.1 mg HA and DHEA-S + 1 mg HA (P < 0.03, P < 0.03, P < 0.02, P < 0.0001 respectively) compared with control. The water content was more significantly increased in cervices treated with DHEA-S + 1 mg HA than DHEA-S + 0.1 mg HA (P < 0.05).

Collagenase activity was significantly increased in rabbits given HA, DHEA-S, DHEA-S + 0.1 mg HA and DHEA-S + 1 mg HA (P < 0.04, P < 0.03, P < 0.01, P < 0.0001 respectively) compared with control. Also, collagenase activity in DHEA-S + 1 mg HA-treated rabbits was markedly increased compared with DHEA-S + 0.1 mg HA (P < 0.009). Gelatinase activity was significantly increased in cervices treated with HA, DHEA-S, DHEA-S + 0.1 mg HA and DHEA-S + 1 mg HA (P < 0.009, P < 0.007, P < 0.002, P < 0.0001 respectively) compared with controls. However, gelatinase activity in DHEA-S + 1 mg-treated cervices was markedly increased compared with DHEA-S + 0.1 mg HA treatment (P < 0.001). Cervical granulocyte elastase activity in HA, DHEA-S, DHEA-S + 0.1 mg HA and DHEA-S + 1 mg HA-treated rabbits was significantly increased (P < 0.03, P < 0.01, P < 0.009, P < 0.0001 respectively) compared with control. Furthermore, this activity was markedly higher in animals treated with DHEA-S + 1 mg HA than in the DHEA-S + 0.1 mg HA group (P < 0.009).

Picrosirius red staining for collagen study
Picrosirius red staining of cervices treated with DHEA-S + 1 mg HA showed that the collagen fibres became thinner and irregularly separated from each other (Figure 4a). The density of collagen was decreased, and interfibrillar spaces were markedly dilated. In contrast, less pronounced changes in collagen structure were detected in cervices treated with HA (Figure 4b) and DHEA-S (Figure 4c). Control cervix with picrosirius red staining (Figure 4d) revealed that collagen fibres were well organized and densely packed as bundles. The collagen fibres were regularly separated one from another, and oriented in an orderly fashion.

Discussion
Cervical ripening is a complex phenomenon that induces a decrease in collagen concentration (Danforth et al., 1974; Kleissl et al., 1978) and an increase in collagen-degrading enzymes (Mochizuki and Tojo, 1978, 1980; Ito et al., 1979; Kitamura et al., 1980; Mori et al., 1981). The present study suggests that vaginal application of DHEA-S + HA induces a pronounced cervical dilatation and ripening in rabbits, together with decreased cervical collagen concentrations and increased neutrophil infiltration in the cervix of pregnant rabbits. Furthermore, massive dilatation of blood vessels and dissolution of collagen network in the cervices treated with DHEA-S + HA mimicked the spontaneous morphological changes that occur just before term.
Figure 3.

Figure 4.
HA is a potent, cell-derived bioactive macromolecule thought to be involved in many cellular functions such as migration, invasion, proliferation, transformation, mitosis and angiogenesis (Toole, 1972; Tomida et al., 1975; Hopwood and Dorfman, 1977; Kundson et al., 1984; West et al., 1985; Brechet et al., 1986). Dilatation of the uterine cervix at parturition is associated with an increase in HA content. It has been reported that circulating HA concentrations increase significantly at parturition (von Maillot et al., 1979; Rajabi et al., 1992; Osmers et al., 1993). An accumulation of HA in the extracellular matrix may result in the observed softening and swelling of the uterine cervix because of its unique viscoelastic properties and its high avidity for water (Rajabi et al., 1992). The uterine cervix is infiltrated with inflammatory cells that produce exaggerated amounts of IL-1β, tumour necrosis factor (TNF) α and IL-8. Evidence suggests that HA stimulates the secretion of IL-8, IL-1β and TNF-α (Hiro et al., 1986; Kobayashi and Terao, 1997; Maradny et al., 1997). IL-1β and TNF-α synthesized by fibroblasts after stimulation by endogenously produced HA could act directly as a positive feedback of further generation of HA. Our recent study demonstrates that HA is an important factor in the process of cervical ripening which is able to stimulate and regulate the biochemical changes occurring in the cervical tissues at term (Maradny et al., 1997).

DHEA-S has been shown to increase in parallel with the progress of pregnancy (Madden et al., 1976), and therefore found to be associated with cervical dilatation in late pregnancy and the induction of labour (Imai et al., 1992). It is well known that successive administration of DHEA-S to pregnant women accelerates cervical ripening, manifested as increases in collagenase in the cervical tissue (Mochizuki et al., 1978; Sasaki et al., 1982; Mochizuki and Maruo, 1985; Zuidema et al., 1986). Parenteral administration of DHEA-S has been used effectively for cervical ripening and labour induction. The proposed mechanism of action is an activation of total collagenolytic activity, resulting in uterine connective tissue remodelling (Maciulla et al., 1998). Our animal study has suggested that DHEA-S acts synergistically with IL-8 to increase collagenase, elastase and gelatinase activities, while decreasing the cervical collagen content (Maradny et al., 1996).

In our recent experiment, DHEA-S-treated cervical tissues and cervical fibroblasts showed an increase in IL-8 concentration and IL-8 receptor population (Kanayama et al., 1998). Thus, DHEA-S may promote the expression of IL-8 receptors in the cervix and increase the affinity of the tissues to bind IL-8. The upregulation of the autocrine system of IL-8 could markedly affect the production and release of IL-8 (Kanayama et al., 1998).

It was also suggested that cervical ripening is associated with an increase in nitric oxide (NO) production (Buhimschi et al., 1996; Ali et al., 1997). Proinflammatory cytokines enhance the expression of the inducible form of NO synthase (iNOS) (Chwalisz et al., 1996). The changes in iNOS mRNA at the end of pregnancy may play a role in the preterm labour and cervical ripening (Ali et al., 1997). NO plays an important role in the regulation of cervical ripening and the management of premature ripening of the cervix (Romero, 1998). Local administration of NO donors can induce cervical ripening in humans and animals (Chwalisz et al., 1997; Thomson et al., 1997). NO donors, whether alone or in combination with other therapies, would seem to represent an exciting new prospect (Calder, 1998), and may therefore be ideal agents for cervical ripening (Norman et al., 1998). It is rather unlikely that

| Table I. Collagenase, gelatinase and elastase activities and water content in cervixes after treatment |
|-------------|-------------|-------------|-------------|-------------|
| HA | DHEA-S | DHEA-S + 0.1 mg HA | DHEA-S + 1 mg HA | Control |
| Collagenasea | 0.70 ± 0.1 | 0.80 ± 0.2 | 0.89 ± 0.2 | 1.7 ± 0.4 | 0.5 ± 0.1 |
| P < 0.04 | P < 0.03 | P < 0.01 | P < 0.001 |
| Gelatinasea | 12.1 ± 2.7 | 13.5 ± 2.7 | 14.2 ± 2.3 | 22.9 ± 2.5 | 6.8 ± 0.8 |
| P < 0.009 | P < 0.007 | P < 0.002 | P < 0.0001 |
| Elastasea | 7.9 ± 1.3 | 9.0 ± 1.2 | 10.1 ± 1.4 | 16.7 ± 1.5 | 4.5 ± 1.5 |
| P < 0.03 | P < 0.01 | P < 0.009 | P < 0.0001 |
| Water content (%) | 84.5 ± 2.7 | 84.1 ± 2.1 | 85.0 ± 3.0 | 89.0 ± 2.5 | 78.1 ± 1.2 |
| P < 0.03 | P < 0.03 | P < 0.02 | P < 0.0001 |

aEnzyme activity expressed as U/100 mg tissue. Values are mean ± SD. 
P-values are given for comparisons with the relevant control value. Statistical significance was achieved for P < 0.05.

Figure 3. Histological changes in cervixes treated with HA (a), DHEA-S (b), DHEA-S + 1 mg HA (c) and control (d). Treatment with DHEA-S + 1 mg HA showed pronounced cervical dilatation and ripening. The density of the collagenous network was markedly decreased, whereas the density of the ground substances and the vascularity were increased. Massive dilatation of blood vessels was seen. The cervix in control rabbit (d) shows the collagenous network lying close together. The connective tissues are compact and the blood vessels small and non-dilated. Less pronounced changes were found in animals treated with HA and DHEA-S. H&E staining; scale bar = 0.05 mm.

Figure 4. Picrosirius red staining of cervixes treated with HA, DHEA-S, DHEA-S + 1 mg HA and control. In DHEA-S + 1 mg HA-treated cervixes (a), the density of collagen was markedly decreased and collagen fibres were irregularly separated from each other. By comparison, less pronounced changes were seen in cervixes treated with HA (b) and DHEA-S (c). In control cervix (d), the collagen fibres were well organized and densely packed as bundles. Picrosirius red staining; scale bar = 0.05 mm.
systemic administration of a NO donor will produce high enough levels in the cervix to exert proinflammatory effects (Chwalisz and Garfield, 1998). NO may represent a final metabolic pathway of cervical ripening which acts by activating metalloproteinases (MMP) and other mechanisms responsible for extracellular matrix remodelling, such as modulation of proteoglycan synthesis (Chwalisz et al., 1997).

Our findings reveal that DHEA-S + 1 mg HA-induced alteration in the biochemical composition and physical properties of the cervix seems to be much more dramatic. Collagenolytic activity derived from the infiltrating neutrophils is thought to play the main role in cervical ripening. The neutrophil infiltration in the cervix treated with DHEA-S + HA may be mediated not only by the concentration of IL-8 induced by DHEA-S or HA in the tissues, but also by the expression of IL-8 receptors with DHEA-S and the affinity of IL-8 to IL-8-stimulated by HA (Kanayama et al., 1998). DHEA-S has a hydrophilic and negative charge because of its sulphate residue, and this may be related to its cell surface binding (Saitoh et al., 1984). Thus, the receptor expression of IL-8 may become stronger when DHEA-S combines with HA. The expression of IL-8 receptors by DHEA-S or HA-induced IL-8 may promote the accumulation of neutrophils in the uterine cervix; thus, the observed effect will be more pronounced.

We conclude that DHEA-S stimulates neutrophil responses to HA-induced IL-8 and plays a role in promoting an increase in neutrophil accumulation, water content, collagenase, elastase and gelatinase activities, and a decrease in collagen concentration in the cervix of rabbits. We suggest that the DHEA-S + HA-induced changes in cervical connective tissue may account for, at least in part, cervical maturation.

References
DHEA-S accelerates HA-induced effects


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