EFFECTS OF DEHYDROEPIANDROSTERONE SULPHATE ON THE PRODUCTION OF COLLAGENASE AND GELATINOLYTIC METALLOPROTEINASE BY RABBIT UTERINE CERVICAL CELLS IN PRIMARY CULTURES

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Cells from pregnant rabbit uterine cervix in primary cultures produced specific collagenase and gelatinolytic metalloproteinase, both in latent forms. The two enzymes were separated by CM-52 cellulose chromatography. Dehydroepiandrosterone 3-sulphate (DHAS) stimulated the production of the latent collagenase by the cells in a dose-dependent manner and the maximal effect, which was about 2-fold of control cultures, was observed when 1×10^{-6} M DHAS was added to the medium containing 10% (v/v) foetal calf serum (FCS). DHAS also stimulated the production of the gelatinolytic metalloproteinase. The significant stimulative effects were also confirmed when the cells were incubated with 1×10^{-7} M DHAS in the medium containing 9% (v/v) dextran-coated charcoal treated FCS and 1% (v/v) FCS. The uptake of [3 H]DHAS by the cells from pregnant rabbits was 5–10-fold greater than that from nonpregnant rabbits. When confluent cultures were incubated with 6.5×10^{-8} M [3 H]DHAS for 2 d, 3.4% and 0.03% of total radioactivity added were accumulated as [3 H]oestradiol-17 β ([3 H]E $_2$) in the medium and cell layer, respectively. Nevertheless, the addition of 1×10^{-9} M and 1×10^{-11} M E $_2$ or dehydroepiandrosterone (DHA) to the confluent cultures caused no significant effect on the collagenase production. These results strongly suggest that the stimulative effect of DHAS on the production of collagenase and gelatinolytic metalloproteinase by the rabbit uterine cervical cells was due to unchanged DHAS and not due to E $_2$ or DHA converted from DHAS.

Keywords — dehydroepiandrosterone sulphate; collagenase; gelatinase; metalloproteinase; cervical ripening; rabbit uterine cervix; cell culture; dehydroepiandrosterone; oestradiol-17 β ; connective tissue

INTRODUCTION

The uterine cervix consists mainly of connective tissue composed of collagen, proteoglycan and structural glycoprotein. It is well known that marked changes in these connective tissue components were observed in the uterine cervix during dilatation and ripening in pregnancy at term.^{1–5)} However, little is known about the detailed mechanisms of hormonal regulation of these changes in connective tissue metabolism.

Dehydroepiandrosterone 3-sulphate (DHAS) is known as an androgen produced by the adrenal glands,⁶⁾ and is changed into oestrogens in human placenta.⁷⁾ Increases in the level of DHAS in baboon serum⁸⁾ and human umbilical cord plasma⁹⁾ were found to parallel gestational age, indicating that DHAS is concerned directly or indirectly with uterine cervical dilatation in late pregnancy. In fact, DHAS administered to pregnant monkeys induced additional cervical dilatation.¹⁰⁾ Recently, Mochizuki and Tojo^{11,12)} have demonstrated that the injection of DHAS

into women at a late gestational age promotes Bishop score and collagenase release into the culture medium of uterine cervical explants. From these facts, they proposed a hypothesis that such action of DHAS are due to oestrogen converted from DHAS *in vivo*. Oestradiol-17 β (E $_2$), however, has been reported to inhibit collagenase activity *in vivo* in rat uterus,¹³⁾ and to show no effect on the biosynthesis of the enzyme in tissue culture.^{14,15)} The mechanism of action of DHAS on cervical ripening, therefore, may not be precisely explained by the above hypothesis.

Authors have already reported that increases in collagenase¹⁶⁾ and alkaline proteinase¹⁷⁾ are important factors which induce dramatic changes in the human uterine cervix during pregnancy at term, and that the administration of DHAS to rabbits of middle gestational age accelerates the cervical ripening and increases in those enzymes.¹⁸⁾ More recently, we have reported that the exposure of DHAS to the rabbit uterine cervical tissue promotes the collagenase pro-

duction in tissue culture, while that of E_2 or dehydroepiandrosterone (DHA) does not, suggesting that DHAS directly shows its hormonal action.¹⁹⁾ In these studies, however, the mechanism of action of DHAS could not be fully clarified since the origin of such enzymes remained unknown and the experiments were carried out in complicated systems such as *in vivo* or tissue culture. In the present study, we first established the primary culture system of rabbit uterine cervical cells which secrete latent collagenase and gelatinolytic metalloproteinase into the medium, and then examined the effects of DHAS on the production of such enzymes, considering the metabolism of DHAS by the cells.

MATERIALS AND METHODS

Materials — Tissue culture plasticwares were purchased from Corning Glass Works, Corning, NY, U.S.A. The following reagents were commercially obtained: Eagle's minimum essential medium (MEM), and calcium- and magnesium-free Hank's balanced salt solution (HBSS(—)) from Grand Island Biological Co., Grand Island, NY, U.S.A.; calcium- and magnesium-free phosphate buffered saline (PBS(—)) from Nissui Seiyaku Co., Tokyo, Japan; foetal calf serum (FCS) and cycloheximide from Boehringer-Mannheim G.m.b.H., Mannheim, W. Germany; collagenase (type I CLS) from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; soya-bean trypsin inhibitor (STI) (type II-S), bovine serum albumin (BSA) (fraction V), trypsin (type III), 4-aminophenylmercuric acetate (APMA), DHAS, DHA, E_2 , oestriol, 5 α -dihydrotestosterone, testosterone, and E_2 3-sulphate from Sigma Chemical Co., St. Louis, MO, U.S.A.; penicillin from Banyu Pharmaceutical Co., Tokyo, Japan; streptomycin sulphate from Meiji Seika, Tokyo, Japan; Norit A and Scintisol EX-H from Wako Pure Chemical Industries, Tokyo, Japan; [1-¹⁴C]acetic anhydride (20 mCi/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K.; [7-³H(N)]DHAS ammonium salt (23.0 Ci/mmol) from New England Nuclear, Boston, MA, U.S.A.; CM-52 cellulose from Whatman, Maidstone, U.K.; Dextran T-40 from Pharmacia, Uppsala, Sweden and 0.25% (w/v) trypsin solution for tissue culture from Chiba Serum Institute, Chiba, Japan. Other reagents used were of analytical reagent grade.

Cell Culture — The uterine cervixes used for

the preparation of primary cell cultures were obtained from Nippon white rabbits of 23 d gestational age (3.5–4.2 kg body weight) or from nonpregnant control rabbits (2.9–4.0 kg body weight).

The uterine cervix was dissected free of other tissues under sterile conditions, rinsed in HBSS(—) containing 0.5% BSA, penicillin (100 U/ml) and streptomycin (100 μ g/ml) until free of blood contamination and cut with scissors into small pieces (approx. 10 mm³). The pieces of tissue (1–1.7 g wet weight) were incubated at 37 °C in 20 ml of 0.2% (w/v) collagenase/HBSS(—) under continuous stirring. After 10 min the contents were allowed to settle for 5 min in an ice-bath and the supernatant was removed and discarded. The remaining undigested tissue pieces were incubated again at 37 °C for an additional 40–60 min in fresh collagenase solution as described above. After this second dissociation, the dispersed cells were filtered through sterile nylon mesh, centrifuged at 200 $\times g$ for 10 min at 4 °C and washed free of exogenous collagenase with 30 ml of HBSS(—) containing 0.5% (w/v) BSA, 0.05% (w/v) STI and antibiotics described above (3 times). The cell pellet was suspended in 30 ml of culture medium (MEM supplemented with 10% FCS and antibiotics described above), filtered again through sterile nylon mesh and centrifuged as above. The final cell pellet was resuspended in culture medium as described above. Cell viability of 70–85% was routinely obtained, as estimated by trypan blue exclusion. The cells were plated at densities of $1.5-2 \times 10^5$ cells/ml in 35 mm dishes, 16 mm diameter culture wells or 25 cm² flasks containing 2, 1 and 10 ml of culture medium described above, respectively, and cultured at 37 °C in 5% CO₂/95% air. After 24 h, the medium was changed to remove the nonadherent cells. Thereafter, the medium was changed every 2 or 3 days. The cells became confluent within 3 to 4 d. Confluent cultures were used for all of the experiments. Steroids and cycloheximide were added to the medium at the indicated concentrations in each experiment as an ethanol solution. The final ethanol concentration was 0.1% (v/v) in all cultures, and the same amount of vehicle was added to the control cultures. The harvested medium was adjusted to pH 7.5 with 1/20th volume of 1 M Tris-HCl/200 mM CaCl₂/3 M NaCl (pH 7.5), and stored at

–20 °C until use.

Preparation of Steroids-Depleted Serum

—Physiological concentrations of steroid hormones are present in the serum-containing media in which the cells are usually grown.²⁰⁾ In fact, 1.2×10^{-7} M DHAS, 1.3×10^{-9} M DHA, and 3.7×10^{-11} M E₂ have been found in the FCS used in the present study. Therefore, endogenous steroids were removed from FCS by the dextran-coated charcoal extraction procedure.^{21,22)} In brief, FCS containing 10 mg/ml of Norit A and 1 mg/ml of Dextran T-40 was incubated for 30 min at 55 °C, and centrifuged at $3000 \times g$ for 10 min at 4 °C to remove the charcoal. The serum was sterilized by filtration and stored at –20 °C until use. Removal of DHAS was monitored by adding a trace amount of [³H]DHAS at the beginning of the procedure and following the decrease in radioactivity to less than 2% after the treatment. The procedure did not extract more than 2% of total proteins. The dextran-coated charcoal treated serum supplemented with 10% FCS, which is designated as DCC-FCS, was used in some experiments.

Collagenase and Gelatinase Assay — Latent collagenase in culture medium was activated with trypsin, *i.e.*, the medium (0.1 ml) was incubated with 50 μ l of trypsin (0.4 mg/ml) in 50 mM Tris-HCl/10 mM CaCl₂/150 mM NaCl (pH 7.5) at 25 °C for 10 min, then 50 μ l of STI (1.8 mg/ml) in the same buffer was added and incubated for 10 min at 37 °C. Latent gelatinase was also activated by the same procedure. Collagenase was assayed by using [1-¹⁴C]acetylated reconstituted fibrils of guinea pig skin collagen as a substrate.²³⁾ One unit of collagenase hydrolyzes 1 μ g of collagen/min under these conditions. Gelatinolytic activity was assayed as described elsewhere²⁴⁾ with slight modifications. [1-¹⁴C]acetylated gelatin (0.2 ml), which was prepared by thermal denaturation of the above reconstituted ¹⁴C-labelled collagen fibrils at 50 °C for 20 min, and the sample (0.1 ml) were mixed and incubated at 37 °C for 13 h. A solution (0.1 ml) of 60% trichloroacetic acid was added to terminate the reaction and centrifuged at $8000 \times g$ for 5 min. The radioactivity in the supernatant (0.1 ml) was counted in Bray's solution (5 ml) with an Aloka liquid scintillation counter model 903. One unit of gelatinolytic enzyme hydrolyzes 1 μ g of gelatin/min under the conditions employed.

Uptake of [³H]DHAS — Cells from nonpregnant or pregnant rabbits were grown in multiwell dishes with MEM containing 10% FCS as described above. At confluence of cells, the medium was removed and washed twice with MEM. Then the cells were labelled with 6.5×10^{-8} M [³H]DHAS in 0.5 ml of MEM containing 10% (v/v) DCC-FCS. After 4 h, the medium was removed and the cells were washed 4–5 times with PBS(–). Then, the cells were solubilized in 1 M NaOH (1 ml/well) at room temperature for 18 h. An aliquot of 0.5 ml was neutralized with 0.5 ml of 1 M HCl, and counted in 10 ml of Scintisol EX-H. Another aliquot of 0.5 ml was neutralized with 0.25 ml of 2 M HCl and used for protein determination by the method of Lowry *et al.*²⁵⁾

For identification of the metabolites of [³H]DHAS, a whole cell binding assay technique²⁶⁾ was adapted with slight modifications. The cells were grown to confluence as described above in 25 cm² flasks, and labelled with 6.5×10^{-8} M [³H]DHAS in 5.5 ml of the medium described above. After 43 h, the medium was removed and pooled. The cells were washed once with 5.5 ml of MEM and the medium was combined with the above pooled medium (medium fraction). The cells were washed twice with 15 ml of PBS(–). The labelled cells were detached using 2 ml of 0.25% (w/v) trypsin–0.02% (w/v) disodium ethylenediamine tetraacetic acid (EDTA·2Na), and then again with 1.5 ml of the same solution. The pooled cell suspension (3.5 ml) was added to the same volume of 20 mM Tris-HCl/1 M KCl (pH 7.4), sonicated 3 times at 4 °C for 15 s (50 W) each, with intervals of 30 s cooling, and allowed to settle for 30 min. Then the suspension was centrifuged at $800 \times g$ for 10 min at 4 °C, and the supernatant was obtained as a cell fraction. Aliquots of the medium fraction and the cell fraction obtained as above were extracted with 1.5 volume of butan-1-ol. The extracts were dried and applied to silica gel thin layer plates (Kieselgel 60F₂₅₄; E. Merk, Darmstadt, Germany) with a small volume of methanol. The plates were developed in chloroform–methanol–water (75:22:3, v/v). Radioactivity was quantitated by counting 0.5 cm sections of the silica in Scintisol EX-H. The recovery of radioactivity from the butan-1-ol layer was over 95%. The purity of [³H]DHAS was 99.3% and the contaminants

were corrected for each metabolites in the samples. [^3H]DHAS ($R_f = 0.27$), DHAS ($R_f = 0.27$), DHA ($R_f = 0.84$), E_2 ($R_f = 0.72$), oestriol ($R_f = 0.52$), 5α -dihydrotestosterone ($R_f = 0.86$), testosterone ($R_f = 0.85$), and E_2 3-sulphate ($R_f = 0.15$), were used simultaneously as standards.

CM-52 Cellulose Chromatography — Culture medium (370 ml) was concentrated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ at 60% saturation. The precipitate collected by the centrifugation at $20000 \times g$ for 20 min was dissolved in 16 ml of 50 mM Tris-HCl/10 mM $\text{CaCl}_2/0.05\%$ (w/v) Brij 35/0.02% (w/v) NaN_3 (pH 7.5) and then dialyzed against the same buffer. The dialyzed material (27 ml) was applied to a CM-52 cellulose column (3×10 cm) previously equilibrated with the same buffer. The column was first washed with the same buffer and then with a linear gradient of NaCl (0–0.4 M) in 300 ml of the buffer at a flow rate of 18 ml/h. Each effluent was activated by incubation with 0.5 mM APMA at 37°C for 30 min, and assayed for collagenase and gelatinolytic activity as described above.

Sodium Dodecyl Sulphate (SDS)-Polyacrylamide-Gel Electrophoresis — The SDS-polyacrylamide-gel electrophoresis was performed by the method of Laemmli²⁷ using a 7.5% (w/v) acrylamide slab-gel in 25 mM Tris/192 mM glycine/0.1% (w/v) SDS (pH 8.3). Gels were stained with Coomassie brilliant blue R.

RESULTS AND DISCUSSION

Synthesis of Collagenase and Gelatinolytic Activity by Rabbit Uterine Cervical Cells in Primary Culture

When the cells from a pregnant rabbit uterine cervix were cultured in MEM containing 10% (v/v) FCS, collagenase and gelatinolytic activities were not detected in the medium until the cells reached confluence. Latent collagenase first appeared in the medium 2 d after the confluence of cells (day 6), reached a maximum on day 10 and continued to be produced maximally for the duration of the experimental period (Fig. 1). The maximal synthesis was maintained for at least 30 d examined (results not shown). Latent gelatinolytic activity was also found in the medium and displayed the same temporal pattern of production, but the activity was about 30% of that of the latent collagenase. Cycloheximide (7 $\mu\text{g}/\text{ml}$) completely prevented the production of

both enzymes within 48 h after addition, indicating that the enzymes were synthesized by the cells during culture. The amounts of the enzymes synthesised by the cells varied slightly with the animals used. In the absence of serum, however, none of the two enzyme activities were found in the medium after confluence of cells. The latent enzymes in the medium containing FCS could be activated by trypsin as described in Materials and Methods, but not by APMA.

It has been observed that the uterine cervixes of pregnancy at term are very congestive and the cervical ripening is often compared with an inflammatory reaction as described by Liggins.²⁸ It is well known that collagenase and gelatinase are contained in polymorphonuclear leucocytes²⁹ and macrophages.^{30,31} Therefore, the origin of collagenase which was increased by the administration of DHAS to rabbits¹⁸ and with the progress of pregnancy (A. Ito, K. Kitamura,

FIG. 1. Synthesis of Collagenase and Gelatinolytic Activity by Rabbit Uterine Cervical Cells in Primary Cultures

The cells from a 23-d pregnant rabbit were cultured in a multiwell dish, and the medium was harvested every two days. On day 10, the wells were divided into two groups. One group was incubated in the presence of 0.1% (v/v) ethanol (control culture), and the other in the presence of 7 $\mu\text{g}/\text{ml}$ of cycloheximide with vehicle (cycloheximide treatment). Aliquots (50 μl) were assayed for collagenase and gelatinolytic activity after activation with trypsin as described in the text. Values are means $\pm$ S.D. of the indicated number (in parenthesis) of the wells. ●, collagenase; ○, gelatinolytic activity; —, control culture; ----, cycloheximide treatment.

S. Hirakawa, Y. Mori unpublished work) was questioned, whether it resides in such leucocytes or in the uterine cervical cells. Observation under a light microscope showed that the population of the cells in the primary culture system was composed mainly of fibroblastic cells (Fig.

FIG. 2. Rabbit Uterine Cervical Cells in Primary Culture

The cells derived from the uterine cervix of 23-d pregnant rabbit on the 5th day in cultivation were stained with Giemsa's solution. Original magnification 50 x.

2). Nonspecific esterase staining for the identification of monocytes and macrophages³²⁾ showed few such cells were present in the cultures. These results indicate that the latent collagenase and the latent gelatinolytic activity are not of leucocyte origin.

Separation of Latent Collagenase and Latent Gelatinolytic Activity and Their Properties

CM-52 cellulose chromatography was performed for the separation of the latent collagenase and the latent gelatinolytic activity. As shown in Fig. 3, the latent collagenase was completely retained on the ion-exchange column, while the latent gelatinolytic activity was not, indicating that the two enzyme activities did not occur as a single enzyme. After chromatography, none of active enzymes were detected in the effluents from the column. However, both enzymes could be activated with APMA. This is mainly due to the conversion of α_2 -macroglobulin in the serum to an inactive form produced by the initial treatment with $(\text{NH}_4)_2\text{SO}_4$.³³⁾

The patterns of collagen digestion by the enzymatically active fraction from the column on the electrophoretogram are shown in Fig. 4. Typical α^A and β^A bands were seen by the

FIG. 3. Ion-Exchange Chromatography of Latent Collagenase and Latent Gelatinolytic Activity in Culture Medium on CM-52 Cellulose

To a pooled culture medium (370 ml), solid $(\text{NH}_4)_2\text{SO}_4$ (60% saturation) was added and the mixture was centrifuged at $20000 \times g$ for 20 min. The pellet was dissolved in 16 ml of 50 mM Tris-HCl / 10 mM CaCl_2 / 0.05% (w/v) Brij 35 / 0.02% (w/v) NaN_3 (pH 7.5) and dialyzed against the same buffer. The dialyzed material (27 ml) was applied to a CM-52 cellulose column (3×10 cm) previously equilibrated with the same buffer. The column was first washed with the same buffer, and then with a linear gradient of NaCl (0–0.4 M) in 300 ml of the buffer at a flow rate of 18 ml/h. Each effluent was activated by incubation with 0.5 mM APMA at 37 °C for 30 min, and assayed for collagenase and gelatinolytic activity as described in the text. ○, absorbance at 280 nm; ●, collagenase; Δ, gelatinolytic activity.

action of collagenase alone (lane 2). More extensive digestion by the mixture of both enzyme fractions showed complete conversion of α^A and β^A bands to smaller fragments (lane 3).

The effects of various reagents on collagenase and gelatinolytic activity of the effluents from the CM-52 cellulose chromatography were examined. Both enzyme activities were completely inhibited by 2 mM EDTA·2Na, 2 mM 1,10-phenanthroline and 5% (v/v) rabbit serum. L-Cysteine (2 mM) (95% and 98% inhibition for collagenase and gelatinolytic activity, respectively) and 2 mM dithiothreitol (69% and 98% inhibition for collagenase and gelatinolytic activity, respectively) were also potent inhibitors. These results indicate that both enzymes are typical metal dependent proteinases.

Effects of DHAS

The time course of the effect of DHAS on the production of collagenase by rabbit uterine cervical cells in primary culture is shown in Fig. 5. Addition of DHAS (1×10^{-6} M) to confluent cultures maintained in MEM containing 10% (v/v) FCS caused about a 2-fold increase in the secretion of collagenase into the medium. This

effect was initially observed 2 d after the treatment, although a little difference in the magnitude or the time-frame of the response of the cells to DHAS was observed in each different culture. The observed stimulation was not due to a change in cell number because 1×10^{-6} M DHAS did not increase the cell number or incorporation of [3 H]thymidine into the cells (results not shown). These results indicate that DHAS is concerned directly or indirectly with the stimulation of collagenase production by the cells. Table I summarizes the effect of DHAS on the production of collagenase and gelatinolytic metalloproteinase by the cells, and also shows the effect of E_2 and DHA, which are known as the main metabolites of DHAS in the pregnant rabbits,³⁴⁾ on collagenase production. When the cells were maintained in MEM containing 10% (v/v) FCS, DHAS stimulated the production of both collagenase and gelatinolytic metalloproteinase in a dose-dependent manner (Table I-I), while E_2 showed no effect on collagenase production in the range of 1×10^{-6} – 1×10^{-15} M (Table I-II). Then, we examined the effects of the steroids using a medium containing 10% (v/v) DCC-FCS, which consisted of 9% (v/v)

FIG. 4. Cleavage of Collagen by Collagenase and Gelatinolytic Activity

The peaks eluted from a CM-52 cellulose column (Fig. 3) were used after activation as the enzyme preparations. Collagenase alone (0.25 units) or a mixture of collagenase (0.25 units) and gelatinolytic activity (0.5 units) were added to the standard assay system as described in the text. Aliquots (10 μ l) of the incubation mixtures were subjected to SDS-7.5% polyacrylamide-slab-gel electrophoresis. Lanes: 1, collagen; 2, collagen + collagenase; 3, collagen + collagenase + gelatinolytic activity.

FIG. 5. Time Course of the Effect of DHAS on Collagenase Production by Rabbit Uterine Cervical Cells

Confluent cultures grown in 35 mm dishes were incubated with (○) or without (●) 1×10^{-6} M DHAS in MEM containing 10% (v/v) FCS. At each time point, the medium was harvested and assayed for collagenase activity as described in the text, and the culturing was continued for a next period with fresh medium in the presence or absence of 1×10^{-6} M DHAS. Each point represents the mean $\pm$ S.D. of the cumulative totals of the collagenase from three dishes.

dextran-coated charcoal treated FCS and 1% (v/v) untreated FCS, in order to reduce the effects of physiological concentrations of endogenous steroids contained in the FCS as described in Materials and Methods. Although complete substitution of the charcoal treated serum for the FCS resulted in extremely low secretion of collagenase, the use of DCC-FCS caused no depression of collagenase secretion and the uptake of [³H]thymidine by the cells as compared with

the use of untreated FCS (results not shown). Addition of 1×10^{-7} M DHAS to the confluent cultures maintained in MEM containing 10% (v/v) DCC-FCS caused significant increase in the production of collagenase and gelatinolytic activity. In contrast, the addition of E₂ and DHA caused no significant effect on collagenase production by the cells at the concentrations employed (Table I-III, IV, and V). The extent of the increase in the enzyme production caused by the

TABLE I. *Effect of DHAS, DHA and E₂ on Collagenase Production by Rabbit Uterine Cervical Cells in Primary Culture*

Experiment	Hormone	(M)	Collagenase activity (units/well)		Gelatinolytic activity (units/well)	
I (FCS)	Control		11.84 ± 1.68	(100)	3.15 ± 0.12	(100)
	DHAS	1×10^{-6}	20.86 ± 1.24 ^{b)}	(180)	4.70 ± 0.10 ^{c)}	(150)
		1×10^{-7}	18.18 ± 3.66		4.29 ± 0.58	
		1×10^{-9}	15.56 ± 2.00		3.24 ± 0.54	
II (FCS)	Control		11.60 ± 1.02	(100)		
	DHAS	1×10^{-6}	19.34 ± 3.46 ^{a)}	(170)		
	E ₂	1×10^{-6}	12.32 ± 0.38			
		1×10^{-9}	12.02 ± 2.24			
		1×10^{-12}	14.42 ± 1.10			
		1×10^{-15}	13.58 ± 1.50			
III (DCC-FCS)	Control		9.30 ± 0.72	(100)	2.52 ± 0.12	(100)
	DHAS	1×10^{-7}	12.82 ± 1.04 ^{a)}	(140)	3.53 ± 0.30 ^{a)}	(140)
		1×10^{-9}	10.24 ± 3.12		2.21 ± 0.39	
		1×10^{-11}	9.18 ± 1.46		2.68 ± 0.17	
IV (DCC-FCS)	Control		11.98 ± 1.36	(100)		
	DHAS	1×10^{-7}	16.44 ± 0.52 ^{a)}	(140)		
	E ₂	1×10^{-7}	13.98 ± 0.64			
		1×10^{-9}	12.02 ± 1.98			
		1×10^{-11}	13.22 ± 1.82			
		1×10^{-13}	10.42 ± 1.66			
V (DCC-FCS)	Control		18.98 ± 0.72	(100)		
	DHAS	1×10^{-7}	22.40 ± 0.68 ^{b)}	(120)		
	DHA	1×10^{-9}	19.46 ± 1.62			
		1×10^{-11}	19.06 ± 0.46			
		1×10^{-13}	16.96 ± 0.78			

Primary rabbit uterine cervical cells from 23 d pregnant rabbits were grown to confluence in MEM containing 10% (v/v) FCS as described in the text. Confluent cultures were incubated in the absence (control) or presence of the indicated concentrations of the steroids in MEM containing 10% (v/v) FCS (I and II) or 10% (v/v) DCC-FCS (III–V), and the medium was harvested and changed to fresh medium with the same treatment every two days. The medium harvested on the 4th day after confluence was assayed for collagenase and gelatinolytic activity after activation with trypsin as described in the text. The data from three different cell preparations of experiment I–III, IV and V are presented. Data are represented as the mean ± S.D. of three wells. a) $p < 0.05$, b) $p < 0.01$, c) $p < 0.001$, significantly different from control. A relative activity represented as the percentage of control is in parenthesis when significant.

addition of DHAS was very similar to that observed in tissue culture system previously reported.¹⁹⁾

The observations described above encouraged us to investigate the uptake of DHAS by the above cells and to compare that with the uptake by the cells derived from nonpregnant rabbits, since we have observed that the amount of directly extracted collagenase in the pregnant rabbit uterine cervix was significantly greater than that in the nonpregnant rabbits (A. Ito, K. Kitamura, S. Hirakawa and Y. Mori unpublished work). As shown in Fig. 6, the uptake of [³H]DHAS by the cells from pregnant rabbits was 5–10-fold greater than that from nonpregnant rabbits. Furthermore, the uptake of [³H]DHAS was inhibited 70–80% by the presence of 200-fold excess unlabelled DHAS, while the inhibition in the case of cells derived from nonpregnant rabbits was only about 35%. These results suggest that the [³H]DHAS was taken up *via* binding to limited numbers of binding sites, and the difference in the binding

FIG. 6. Uptake of [³H]DHAS by Rabbit Uterine Cervical Cells

Uterine cervical cells from nonpregnant (a-I, II), 23-d pregnant (b-III) and 22-d pregnant (b-IV) rabbits were cultured to the confluence in multiwell dishes. Then, the confluent cultures (about 126 μ g cell protein/well) were incubated with 6.5×10^{-8} M [³H]DHAS in MEM containing 10% (v/v) DCC-FCS in the absence (□) or presence of 200-fold excess unlabelled DHAS (I–IV; ■), E₂ (IV; ▨) and DHA (IV; ▩). After 4 h, the cells were solubilized in 1 M NaOH and the radioactivity was quantitated. Other conditions are described in the text in detail. Results are presented as means $\pm$ S.D. for three (I, II, and III) or four (IV) wells.

sites reflected collagenase production by non-pregnant and pregnant rabbits. However, the inhibition of the uptake of [³H]DHAS was also observed by the addition of the excess amounts of unlabelled E₂ (46% inhibition) or DHA (54% inhibition). Next, we investigated the metabolism of [³H]DHAS by the cells derived from pregnant rabbits to identify the actual contributor for the stimulation of the syntheses of collagenase and gelatinolytic metalloproteinase and for the binding of DHAS to the cells. When confluent cultures were incubated with 6.5×10^{-8} M [³H]DHAS in MEM containing 10% (v/v) DCC-FCS for 2 d, [³H]E₂ was found as the only metabolite in both medium and cell fractions, which was only 3.43% of total radioactivity added (Table II). DHA could not be detected under the condition employed, suggesting that the sulphatase activity was extremely low and/or the aromatase activity was so high that DHA was not accumulated. In the cell fraction, however, 52.4% of the radioactive materials was found to be composed of the [³H]E₂, which might cause the inhibition of total uptake of the radioactivity as seen by the addition of excess unlabelled E₂ shown in Fig. 6 (IV). It is well known that estrogen receptors are present in the human uterine cervix.³⁵⁾

These results indicate that the incubation of 1×10^{-7} M DHAS with the cells for 2 d may result in the formation of about 3×10^{-9} and 3×10^{-11} M E₂ in the medium and the cell, respectively. Under the same condition, about 3×10^{-9} and 3×10^{-11} M DHA in the medium and the cell, respectively, must be formed as an intermediate form, since the formation of E₂ requires the initial action of steroid sulphatase on DHAS. However, the addition of 1×10^{-9} M or 1×10^{-11} M E₂ and DHA to the culture medium showed no effect (Table I–IV and V). Thus, this strongly suggests that the stimulative effect of DHAS on the production of collagenase and gelatinolytic metalloproteinase by the cells is not due to E₂ or DHA converted from DHAS, but due to DHAS itself.

General Discussion

In the present study, we have been able to demonstrate that the cells from the pregnant rabbit uterine cervix produce typical latent collagenase, and that the collagenase is not of macrophage or granulocyte origin. It has also been demonstrated that the cells synthesize gelati-

TABLE II. Conversion of [^3H]DHAS to [^3H]E₂ by Rabbit Uterine Cervical Cells

Fraction	Radioactivity in each fraction (dpm/fraction)	[^3H]E ₂ in each fraction (% of total radioactivity in each fraction)
Medium	1.4×10^7	3.4 (3.4)
Cell	7.2×10^3	52.4 (0.03)

Uterine cervical cells from a rabbit of 23 d pregnant were cultured to confluence in 25 cm² culture flasks. Then, the confluent cultures were incubated with 6.5×10^{-8} M [^3H]DHAS in MEM containing 10% (v/v) DCC-FCS. After 43 h, the medium was removed and the cells were detached with 0.25% (w/v) trypsin/0.02% (w/v) EDTA. The cells were sonicated in 10 mM Tris-HCl/0.5 M KCl (pH 7.4) at 4°C and centrifuged at $800 \times g$ for 10 min at 4°C. The medium and cell fractions were extracted with butan-1-ol and the radioactive materials in the extracts were identified by thin-layer chromatography on silica gel plates with a solvent system of chloroform-methanol-water (75:22:3, v/v). Other experimental details are described in the text. Values are given as the average for two separate cultures. Percentage of total radioactivity in a flask is given in parenthesis.

nolytic activity, although the enzymatic properties have not been fully characterized as yet. The cell culture system, therefore, can be used to evaluate the effect of steroid hormones on the cervical ripening in the rabbit in a more simplified system than *in vivo* or tissue culture systems.

We have already reported that E₂ and DHA significantly depressed collagenase production in tissue culture.¹⁹⁾ Recently, Wallis and Hillier³⁶⁾ reported that, in nongravid human uterine cervix cultures, the release of hydroxyproline-containing peptide is significantly inhibited by the addition of E₂ to the culture medium. Similar results for ovine cervix have been reported by Ellwood *et al.*³⁷⁾ However, all of these results were obtained using tissue culture systems which consisted of various types of cells. In contrast, E₂ and DHA showed no significant effects on the collagenase production by rabbit uterine cervical cells as described above.

It is well known that DHAS is converted to DHA and testosterone by the actions of sulphatase and Δ^4 -3 β -hydroxysteroid dehydrogenase (3 β -HSD) and then changed to estradiol by the action of aromatase, and that human placenta contains high levels of 3 β -HSD and aromatase.⁷⁾ In fact, the administration of DHAS to pregnant women induced a significant increase in the concentration of E₂ in serum and portio vaginalis.¹²⁾ Similar results are also reported with pregnant monkeys.¹⁰⁾ On the other hand, negligible amounts of 3 β -HSD and aromatase were detectable in rabbit placenta.³⁸⁾ It is also reported that after administration of DHAS to late pregnant rabbits, serum levels of DHAS, DHA, testosterone and E₂ increased gradually,

and reached a maximum level 30 min later, but the increases in DHA and testosterone levels were only 0.1% of the administered DHAS and that of E₂ was about 0.0001%.³⁴⁾ However, it is more important to learn the local metabolism of DHAS by the cells in order to elucidate the mechanism of DHAS action. The results in the present study (Fig. 6 and Table II) indicated that about 50% of intracellular metabolites which were accumulated by the incubation of DHAS with the cells for 2 d were E₂. Nevertheless, E₂ or a possible intermediate DHA showed no effect on the collagenase production. Thus, it is suggested that the effect of DHAS on cervical ripening in the rabbit is, at least partially, due to unchanged DHAS, although it still remains unknown whether the action of DHAS is mediated *via* mechanisms consistent with the general concept of steroid hormone action, *i.e.*, hormone-receptor interaction.

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