



Atheroprotective effect of estriol and estrone sulfate on human vascular smooth muscle cells

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Abstract

In patients with atherosclerosis, fibrosclerotic focuses are induced by multiplication of vascular smooth muscle cells (VSMC), and they are regulated by cytokines and regulators. There have been few reports about the atheroprotective effect of estriol (E_3). Estrone sulfate (E_1 -S) is the predominant estrogen of conjugated equine estrogens, which is commonly used in hormone replacement therapy, but it should be hydrolyzed by steroid sulfatase (STS) to enter the cells of target tissues. The purpose of this study was to detect STS in VSMC and to investigate whether E_3 and E_1 -S have atheroprotective effects like E_2 . First, we detected the presence of STS mRNA in VSMC by in situ hybridization. We then examined the changes in the expression of mRNAs of cytokines, namely, PDGF-A chain, IL-1, IL-6 and TGF- β , in VSMC, in the presence and absence of E_3 and estrogens. As a result, the expression of PDGF-A chain, IL-1 and IL-6 mRNAs was suppressed by E_3 ($P < 0.05$ vs control) significantly like E_1 -S and E_2 , but that of TGF- β mRNA was not significantly affected by any estrogen. These results indicate that E_1 -S can be hydrolyzed by STS in VSMC, and that E_3 may regulate the cytokines by suppressing the production of mRNAs. It is suggested that there is a possibility of E_1 -S and E_3 having a direct effect on vessels in atherogenesis. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Estriol (E_3) is recognized as a ‘weak estrogen’, while estradiol (E_2) is recognized as a ‘strong estrogen’. In Japan, however, E_3 is widely used in clinical practice, not only in gynecology, but also in internal medicine and orthopedics, because it has proved useful in the treatment of osteoporosis in patients without endometrial proliferation [1]. Indeed, it has been suggested that hormone replacement therapy (HRT) is necessary to prevent osteoporosis [2,3] and atherosclerotic disease [4,5], as well as improving the vasomotor system and treating urogenital dystrophy.

In recent years, the direct atheroprotective effect of estrogens on vascular cells [6–8] has attracted much attention. In patients with atherosclerosis, fibrosclerotic focuses are induced by multiplication of smooth muscle cells. They are regulated by paracrine and auto-crine factors such as Platelet-derived growth factor (PDGF) [9], interleukin-1 (IL-1) [10], interleukin-6 (IL-6) [11] or transforming growth factor- β (TGF- β) [12]. We have previously shown that estrogens suppressed the induction of growth factors on vascular cells [13], but there have been few reports about the direct effect of E_3 on vascular cells. The purpose of this study was to detect STS in VSMC and to investigate whether E_3 and E_1 -S have atheroprotective effects like E_2 .

Conjugated equine estrogen (CEE), whose main component is E_1 -S, is used extensively for HRT. We mainly compared the effect of E_3 with E_1 -S. However,

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sulfated estrogens must be hydrolyzed in situ by steroid sulfatase (STS) to unconjugated estrogens so as to enter the cells of target tissues [14]. For this reason, first we measured the level of STS mRNA in VSMC by in situ hybridization (ISH). We then examined and discussed changes in the expression of mRNAs of growth regulatory molecules, namely PDGF-A chain, IL-1, IL-6 and TGF- β , in VSMC in vitro, in the presence or absence of E₃, E₁-S and E₂.

2. Materials and methods

2.1. Detection of STS mRNA by in situ hybridization

2.1.1. Cell culture and fixation

Human vascular smooth muscle cells (HASMC) (Kurabo Co., Osaka, Japan) were cultured on chamber slides (Lab-Tek chamber slide, Nunc, IL, USA) to sub-confluency in HuMedia-SG2 medium (Kurabo Co.), at 37°C in a 5% CO₂ atmosphere. The cells were washed with phosphate-buffered saline (PBS), pH 7.4 for 1 min, fixed with 100% ethanol for 1 min, and hydrated by passing the slides through a series of ethanol solutions (70%, 50%, then 30% ethanol), for 5 min in each solution. Then they were treated with PBS containing Protease K (1 μ g/ml) for 10 min at 37°C, and postfixed with PBS containing 4% paraformaldehyde for 10 min at room temperature (RT). The cells were then washed twice with PBS containing 5 mM MgCl₂ and air-dried.

2.1.2. Probe preparation

Cloning of the cDNA fragment of steroid sulfatase (STS) [15] was carried out using the reverse transcriptase and PCR techniques. The cDNA fragment of STS was amplified from genomic DNA of human placenta. The amplified length and primers used were as follows: STS 275bp: 5'-GAACACTGAGACTCCGTTTCCT-3', 5'-CTTTATAGATCCCATTACTTCCGCC-3'. Amplified cDNA fragments were inserted at the Sac II site of pGEM-T[®] (Promega, USA) and its sequences were verified using a sequence analyzer. Dig-labeled RNA probes were prepared employing Dig RNA labeling kit (Boehringer Mannheim, Germany). Antisense probes were transcribed by Sp6 RNA polymerase using Nco I-digested plasmids as templates, and sense probes were transcribed by T7 RNA employing Spe I-digested plasmids. Both were dissolved in diethylpyrocarbonate (DEPC) treated distilled water and stored at -80°C.

2.1.3. In situ hybridization

Hybridization was performed at 55°C overnight in a moisture chamber containing 50% formamide/2 \times SCC. The hybridization mixture contained the RNA probe (1–2 μ g/ml), 20 mM Tris-HCl, pH 8.0, 25 mM

EDTA, 300 mM NaCl, 1 \times Denhardt's solution, 1 mg/ml *E. coli* transfer RNA (tRNA), 50% deionized formamide and 10% dextran sulfate. A piece of parafilm was placed over the reaction mixture to prevent evaporation during hybridization. The parafilm was removed in 5 \times SCC at 42°C, and the hybridized sections were washed three times with 50% formamide/2 \times SCC at 42°C for 20 min each time, and then treated with RNase A (10 μ g/ml) for 30 min at 37°C. The sections were washed three times with 0.1 \times SCC at 42°C for 20 min each time and then treated with blocking solution for 1 h; i.e., 1% BSA and 1% blocking reagent (nucleic acid detection kit, Boehringer Mannheim) dissolved in 100 mM Tris-HCl, pH 7.5 containing 150 mM NaCl, followed by incubation with alkaline phosphatase labeled anti-Dig Fab fragment (ALP-anti-Dig Fab: Boehringer Mannheim) for 2 h at RT. The sections were washed with 100 mM Tris-HCl, pH 7.5, containing 150 mM NaCl three times for 15 min each time, and finally with 100 mM Tris-HCl pH 9.5, containing 100 mM NaCl and 50 mM MgCl₂ for 15 min. ALP enzyme activity was detected using 250 μ g nitroblue tetrazolium in the dark, and 175 μ g 5-bromo-4-chloro-3-indoxyl phosphate was dissolved in 1 ml of 100 mM Tris-HCl pH 9.5, containing 100 mM NaCl and 50 mM MgCl₂. After color development, the sections were rinsed in 10 mM Tris-HCl pH 7.6, containing 1 mM EDTA and mounted in the same buffer containing 50% glycerin.

2.2. Quantitative analysis of mRNAs of growth regulatory molecules

2.2.1. Cell culture

VSMC from the 3rd to 5th generation, were cultured to sub-confluency (24,000 cells/cm²) in 25 cm² flask in HuMedia-SG2 medium, at 37°C in a 5% CO₂ atmosphere. Before estrogen was added, they were incubated with phenol red minus MEM (Eagle's MEM "Nissui", Nissui Pharmaceutical Co., Tokyo, Japan) containing 2% DCC treated fetal bovine serum (FBS) for 24 h. E₃, E₁-S, E₁ or E₂ (10⁻¹⁰ M, 10⁻⁸ M) was added and the cells were incubated for 6 h.

2.2.2. Isolation of total cellular RNA

Total RNA was isolated from cells according to the acid guanidium thiocyanate-phenol-chloroform extraction method [16]. Cells were homogenized in 4 M guanidine isothiocyanate (pH 7.0) including 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, and 2 M sodium acetate (pH 4.0), phenol and chloroform-isoamyl alcohol mixture (49:1). The homogenate was vigorously shaken for 10 s and then cooled on ice for 15 min. The samples were centrifuged at 10,000 g for 20 min at 4°C. The precipitated RNA was dissolved in 4 M guanidine isothiocyanate (pH 7.0) containing 0.5% sarco-

syl and 0.1 M 2-mercaptoethanol. The solution was transferred to a microtube and precipitated with the same volume of isopropanol at -20°C for 1 h. After centrifugation at 10,000 *g* for 10 min at 40°C , the RNA pellet was resuspended in 75% ethanol and centrifuged again. The last pellet was dried under vacuum and dissolved in a proper volume of DEPC-treated water.

2.2.3. Reverse transcription polymerize chain reaction (RT-PCR)

Total RNA (5 μg) was reversed transcribed to first strand cDNA using random hexamers (50 ng/ μl) and SUPER SCRIPT II RT (200 units/ μl) by the SUPER SCRIPT Preamplification System (Gibco BRL, Gaithersburg, MD). The cDNAs were synthesized as PCR products after 30 cycles of 45 s at 94°C for denaturation, 45 s at 60°C for annealing, and 2 min at 72°C for extension. PCR was carried out with the reverse transcribed cDNAs (5 μl), Taq polymerase (2.5 U) and 0.1 μM specific primers of growth factors (Table 1) in PCR buffer (0.1 mM dNTP mix and 2.25 mM MgCl_2 [Gibco BRL]).

2.2.4. Southern blot analysis of growth factors of mRNAs

The PCR products were subjected to electrophoresis using 3% agarose gel (NuSieve, FMC Bio Products, USA) at 40 V. PCR products were transferred to a nucleic acid transfer membrane (Hybond-N+, Amersham, Buckinghamshire, England) for 16 h, which was dried at RT. PCR products on the membrane were prehybridized in a buffer containing $6 \times \text{SSC}$, 10 mM EDTA pH 7.5, $2 \times \text{Denhardt's}$ reagent, 100 $\mu\text{g}/\text{ml}$ ssDNA and 0.5% sodium dodecyl sulfate (SDS) for at least 30 min at 55°C , and then hybridized in the same solution with the oligonucleotide probes, synthesized from the sequences of the said genes between the specific primers ($5 \times 10^5 \sim 10^6$ cpm/ml) and radio-labeled with [γ 32P] ATP overnight at 55°C . The mem-

brane was washed in $2 \times \text{SSC}$ containing 0.05% SDS for 30 min at 55°C . The Imaging Plate TYPE BAS-III (Fuji imaging plate, Fuji Photo Film Co., Japan) was exposed to the membrane for 30 min to detect the specifically hybridized blots. The quantification of hybridized mRNA was carried out with BAS 2000 (Bio imaging analyzer, Fuji Photo Film Co.) and then analyzed by calculating the positive image of their blots.

The statistical procedure was an unpaired *t* test comparing the mean of each estrogen-treated group with the mean of the corresponding controls.

3. Results

3.1. In situ hybridization

The signals of hybridized STS mRNA were strongly detected in the cytoplasm of VSMC. In the control experiment using the sense RNA probe, no ALP reaction products were recognized in VSMC (Fig. 1). The abundant existence of STS would have a profound influence on the cell response to conjugated estrogens.

3.2. The expression of mRNAs of growth regulatory molecule

Urabe et al. [13] demonstrated that the optimum conditions for the analysis of small amounts of mRNA, like that of growth factors, were a cycle number of 30–35. In the previous study [13] we measured the PCR products of β -actin, G3PDH and STS. While β -actin and G3PDH are generally used as housekeeping genes, they were affected by estrogen. Moreover, there is the study that β -actin is increased in synthetic type of VSMC [17]. STS was, however, not affected by estrogen and expressed abundantly, so we used it as an internal control. Thus, the results are presented as rela-

Table 1
Specific primers of cytokines and growth factors

| Name | Sequence ^a | Size (bp) ^b |
|--------------|--|------------------------|
| PDGF-A | (U)5'AGAAGTCCAGGTGAGGTTAGAGGAGCAT3' (D)5'CTGCTTCACCGAGTGCTACAATACTTGCT3' | 304 |
| IL-1 | (U)5'CAAGGAGAGCATGGTGGTAGTAGCAACCAACG3' (D)5'TAGTGCCGTGAGTTTCCCAGAAGAAGAGGAGG3' | 491 |
| IL-6 | (U)5'ATGAACCTCTTCCACAAGCGC3' (D)5'GAAGAGCCCTCAGGCTGGACTG3' | 628 |
| TGF- β | (U)5'GCCCTGGACACCAACTATTGCT3' (D)5'AGGCTCCAAATGTAGGGGCAGG3' | 161 |

^a (U): Upstream, (D): Downstream.

^b Expected size of the PCR product, based on the human cDNA sequence.

tive changes in the expression of each mRNA to STS mRNA.

In general, PDGF, which is concerned with the transformation of the VSMC phenotype, induces normal cells to transform into abnormal proliferative cells. In this study, we examined the A chain of PDGF which is composed of two subunits, A and B chain polypeptide. Our results showed that the mRNA levels of PDGF-A chain decreased to 71.5% and 50.9% in the presence of 10^{-10} M and 10^{-8} M E_3 , respectively ($n = 12$, $P < 0.05$ vs control, dose-dependence was not significant). However, the effects were milder than those of other estrogens (Fig. 2).

IL-1 mRNA levels were significantly decreased to 26.8% and 27% in the presence of 10^{-10} M and 10^{-8} M E_3 , respectively ($n = 12$, $P < 0.05$ vs control, dose-dependence was not seen). The effects of E_3 were stronger than those of E_1 -S and E_2 (Fig. 3).

IL-6 mRNA levels decreased significantly ($n = 12$, $P < 0.05$ vs control) to 42.8% and 60.1% in the presence of 10^{-10} M and 10^{-8} M E_3 (Fig. 4), and those of E_1 -S and E_2 also decreased significantly. IL-1 and IL-6, which are autocrine factors, induce the migration of VSMC.

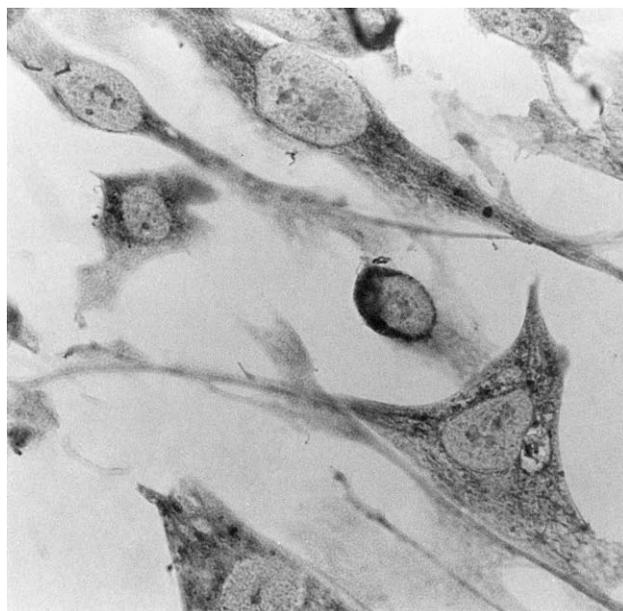
On the other hand, the mRNA level of TGF- β , which is a bifunctional regulator and suppresses migration and proliferation of VSMC in vitro, did not

significantly change when cells were incubated in the presence of any of the estrogens tested in this study (Fig. 5).

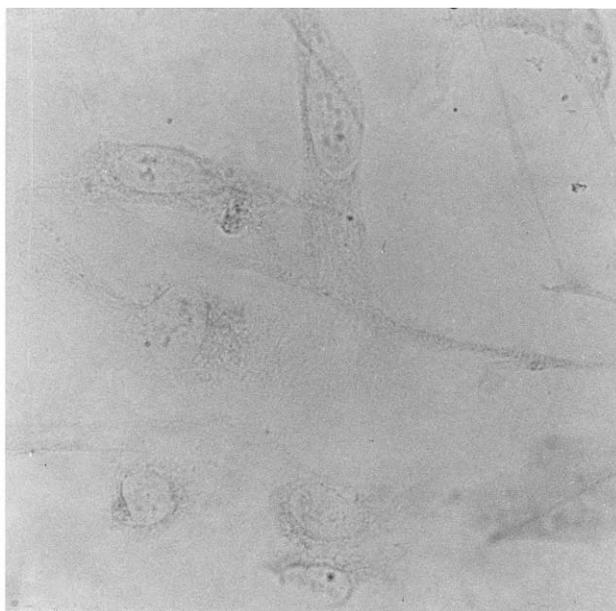
4. Discussion

As HRT has become more well known and has turned out to be even more useful than first thought, estrogen has become widely used in clinic. But in Japan, it is difficult for menopausal women to accept the menses-like hemorrhage associated with HRT, and many professionals except gynecologists can not manage patients with genital bleeding. Therefore, E_3 , which has weaker estrogenic effects than other estrogens, is prescribed to many patients requiring HRT. In recent years, there have been some studies on the effects of estriol on bone loss [1,18]. Persson et al. [19] noted that the risk of endometrial cancer after treatment with estriol alone did not increase as well as it was given together with E_1 -S and progestogens. They suggested that estriol could be safely administered long term. Concerning the lipid effect, it has recently been shown that high doses of E_1 , E_2 and E_3 inhibit oxidation of LDL [20]. However, as far as we know, there is no report of the direct effect of E_3 on vessels in atherogenesis.

Although E_1 was absorbed more rapidly than E_1 -S,



A



B

Fig. 1. Expression of STS mRNA in VSMC. Cultured VSMC were hybridized in situ with antisense or sense probes specific for STS mRNA. (A) Positive signals can be observed in VSMC with the antisense probe. (B) No signal is observed on a control section with the sense probe.

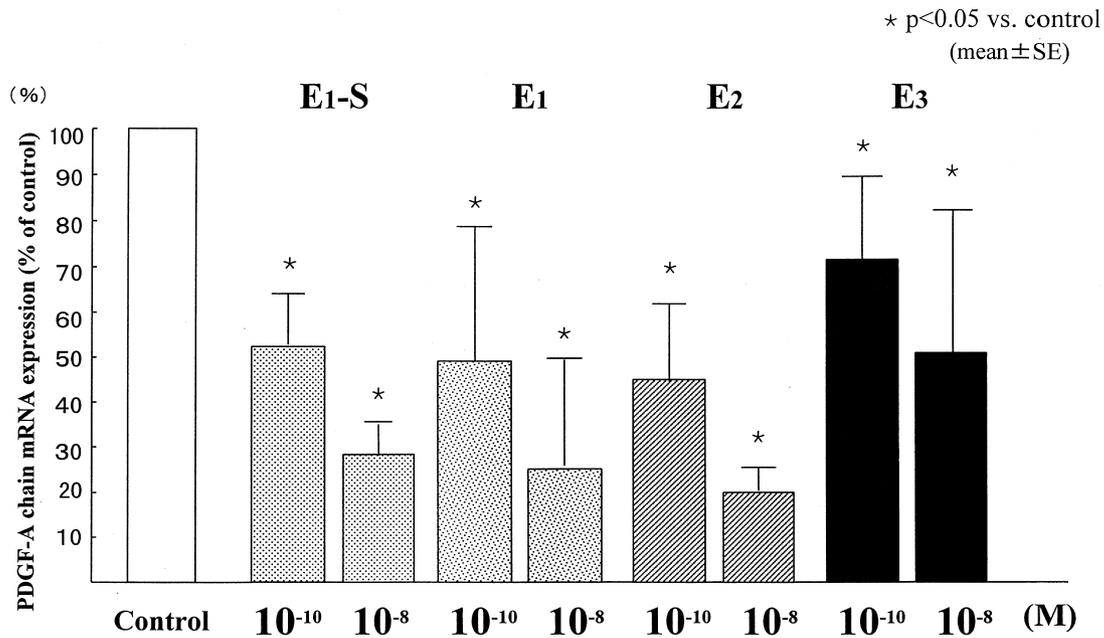


Fig. 2. Effects of E₃ and other estrogens on PDGF-A chain mRNA expression in VSMC. PDGF-A chain mRNA levels decreased to 71.5% and 50.9% in the presence of 10⁻¹⁰ M and 10⁻⁸ M E₃, respectively (*P* < 0.05); however the effects of E₃ were milder than those of other estrogens (*n* = 12 for E₁-S and E₃ administrated group; *n* = 3 for E₁ and E₂ administrated group).

after absorption, E₁ was rapidly sulfated, most likely during the first pass through the liver. E₁-S is the main circulatory form of estrone [21,22]. However, the sulfated estrogens are presented only in the extracellular compartment. Chetrite and Pasqualini [23] indicated that the sulphotransferase enzyme is transported to the cell membrane by an unknown mechanism, and

the sulfated forms are excreted to the medium in vitro. On the analogy of it, E₁-S may be hydrolyzed to E₁ by STS on the cell membrane to enter the cells. The localization of STS was shown at the plasma membrane by immunocytochemistry [24].

E₁-S is known to be hydrolyzed to E₁ by sulfatase, and converted to 17β-estradiol by 17β-hydroxysteroid

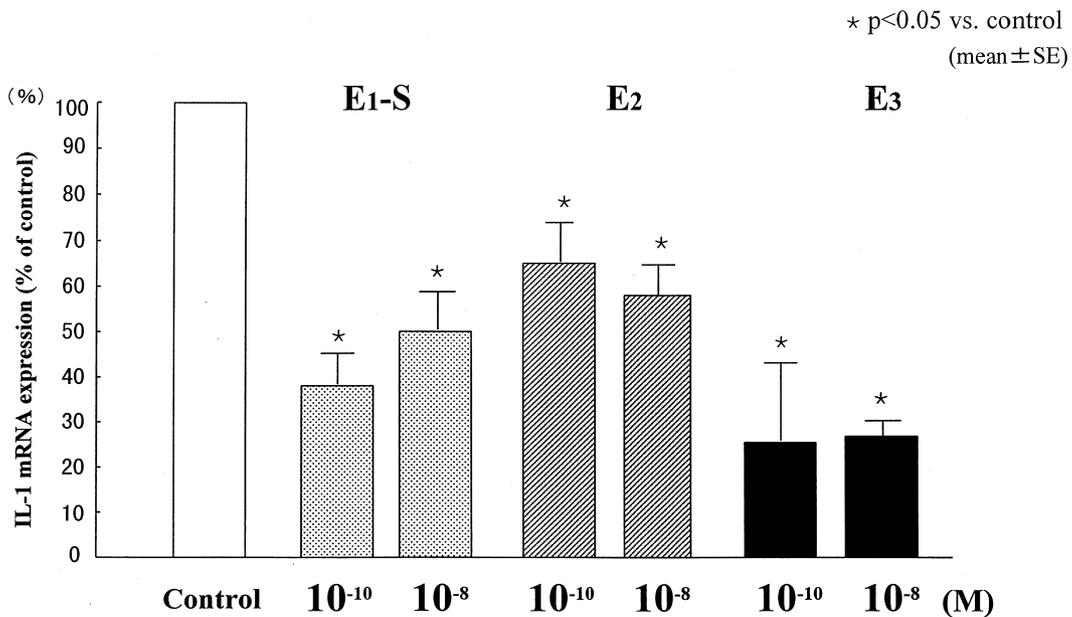


Fig. 3. Effects of E₃ and other estrogens on IL-1 mRNA expression in VSMC. IL-1 mRNA levels significantly decreased to 26.8% and 27% in the presence of 10⁻¹⁰ M and 10⁻⁸ M E₃, respectively (*P* < 0.05). The effects of E₃ were stronger than those of E₁-S and E₂ (*n* = 12 for E₁-S and E₃ administrated group; *n* = 3 for E₂ administrated group).

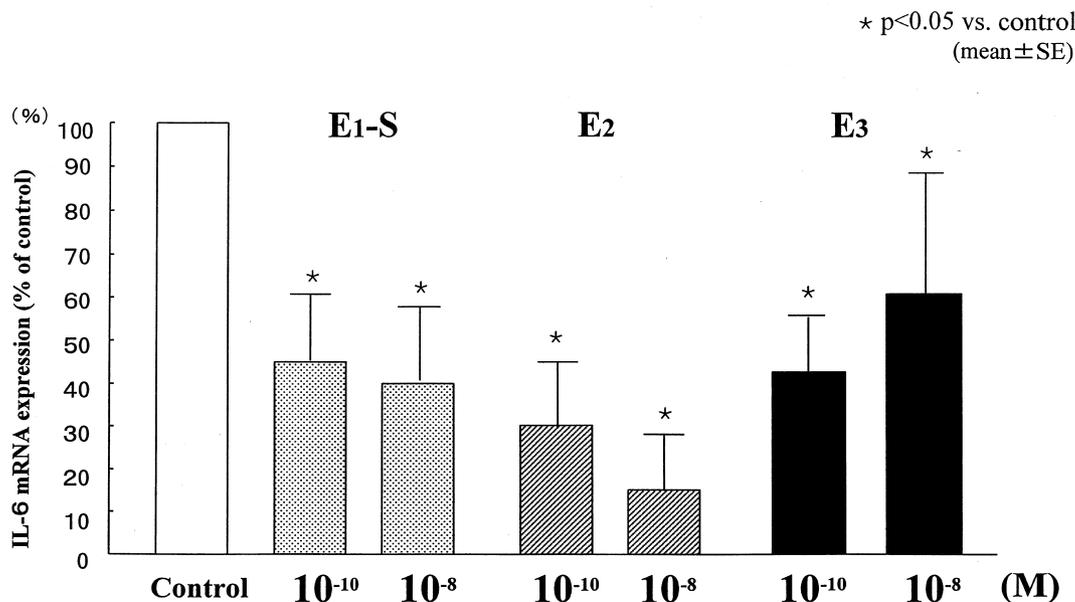


Fig. 4. Effects of E₃ and other estrogens on IL-6 mRNA expression in VSMC. IL-6 mRNA levels were not suppressed in a dose-dependent manner and, although a significant decrease was observed, the effects of E₃ were milder than that of E₁-S and E₂ ($n = 12$ for E₁-S and E₃ administered group; $n = 3$ for E₂ administered group).

dehydrogenase (17 β -HSD). The transfer constants for the conversion of estrone sulfate to estrone and 17 β -estradiol were 0.15–0.21 and 0.014–0.03, respectively, and estrone to 17 β -estradiol was 0.05–0.07 [25,26]. It suggests that E₁-S is easily converted not only to E₁, but also to E₂.

In this study, we have demonstrated by ISH that STS mRNA is found in VSMC. Although further

examination is needed about the metabolism of E₁-S, the existence of STS may indicate that E₁-S can be hydrolyzed into E₁, an active hormone, in target tissues in vivo and regulate both the production of, and the response to, cytokines.

Some authors detected the estrogen-binding site in VSMC [27,6] and we reconfirmed the expression of the estrogen receptor (ER) mRNA by RT-PCR technique

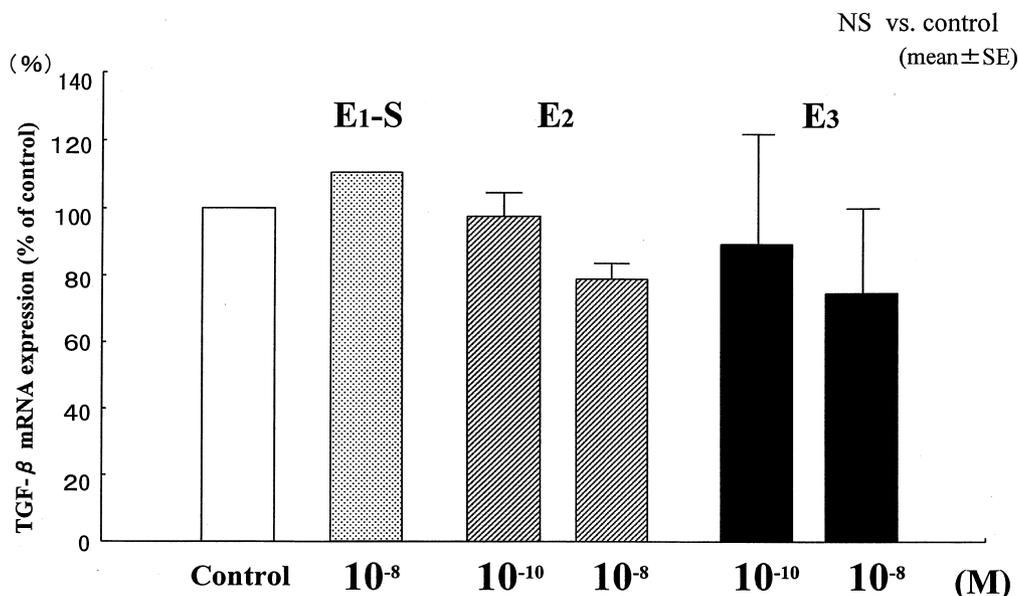


Fig. 5. Effects of E₃ and other estrogens on TGF- β mRNA expression in VSMC. TGF- β mRNA levels did not change significantly after cells were incubated in the presence of any of the estrogens tested ($n = 12$ for E₁-S and E₃ administered group; $n = 3$ for E₂ administered group).

[13]. The existence of STS and ER in VSMC indicates that estrogen can exert direct effects on vascular endothelial cells.

In atherosclerosis, fibrosclerotic focuses are induced by multiplication of VSMC [28]. Their migration and proliferation are regulated by paracrine factors the same as platelets, endothelial cells and macrophages, or by autocrine factors secreted by themselves. In this study, we examined autocrine factors in VSMC and discussed whether E₃ and E₁-S had atheroprotective effects like other estrogens.

The mRNA levels of PDGF-A chain decreased in cells treated with E₃, although its effects were milder than those of E₁-S and other estrogens. PDGF is an important promoter of atherosclerosis, because it induces the transformation of VSMC from contractile cells to synthetic cells, which actively migrate and proliferate in the intima [29,30]. The mRNA levels decreased with E₁-S, the same as E₁, which may indicate that E₁-S has an effect on cells after hydrolyzed to E₁. However, as mentioned previously, it needs more examination about metabolism of E₁-S itself to prove the possibility.

The mRNA levels of IL-6 and IL-1, which induce migration and proliferation of VSMC in vitro, also decreased in cells treated with E₃ and E₁-S. IL-6 acts both as an inflammatory and immune mediator locally, in the vessels, while IL-1 induces changes of the endothelial frame and expands the spaces between cells, which are focuses of edema or cell infiltration [31]. The results showed that the mRNA levels of IL-1 tended to be more strongly suppressed by E₃ than by E₁-S and E₂. While IL-1 induces the proliferation of VSMC, it suppresses the proliferation of endothelial cells [32–34]. It remains uncertain that strong suppression of IL-1 really has an atheroprotective effect.

On the other hand, the mRNA levels of TGF- β , which is a bifunctional regulator and suppresses the migration and proliferation of VSMC in vitro, were not significantly affected in our study. The function of TGF- β is complex in vivo as it works as a suppressor or a promoter, depending on its concentration and the density of cells [35]. It has been reported that TGF- β inhibited the expression of the adhesion protein vascular cell adhesion molecule-1 (VCAM-1), which appears to act as inhibitor of inflammatory responses [36].

Our results indicated that estrogens including E₃ and E₁-S, regulate the growth regulatory molecules in VSMC, thereby inhibiting the early initiation and progression of atherosclerosis. But our results showed only the effects on individual cytokines and growth factors in vitro. In fibrosclerotic focuses, those factors conform a cytokine network and suppress or promote other factors. For example, TGF- β and IL-1 promote the production of PDGF. The influence of estrogens on its network in vivo has yet to be clarified.

Until now, the mechanism of the action of E₃ has not been elucidated in detail. Esposito [37] showed that the existence of estriol in the nucleus is shorter than that of estradiol and this constitutes the major difference between them. Our results showed that E₃ might have atheroprotective effects by suppressing growth factors in vascular cells, the same as E₁ and E₂. But whether it works in vivo just the same as in vitro, remains 'unanswered', because E₃ competes for estrogen receptors with other estrogens, but has a higher affinity for albumin than E₂ in vivo [38]. There are some clinical reports that a uterotrophic effect similar to that of estradiol can be obtained by repetitive injection or vaginal administration of E₃ [39]. Using these administration routes, the effects of E₃ are expected to be stronger than when orally administered. The direct effects of E₃ on vessels in vivo should be further examined in the future. Moreover, clinical studies of E₃ regarding its atheroprotective effects should be encouraged.

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