

## Estrogenic Xenobiotics Increase Expression of SS-A/Ro Autoantigens in Cultured Human Epidermal Cells

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**SS-A/Ro autoantibodies are detected at high levels in patients with autoimmune diseases such as systemic lupus erythematosus. It has been reported that natural estrogen is capable of inducing cell surface expression of SS-A/Ro autoantigens in human epidermal keratinocytes. In this study, we analysed, by reverse transcriptase polymerase chain reaction and immunohistochemistry, the effects of estrogenic xenobiotics (i.e. environmental estrogens) on the expression of 52-kDa SS-A/Ro autoantigen in cultured keratinocytes. At a concentration of 10 µM, various estrogenic xenobiotics derived from plants, insecticides, or detergents induced up to a 3-fold increase in 52-kDa SS-A/Ro mRNA levels in human keratinocytes compared with untreated cells. The immunohistochemistry results paralleled the reverse transcriptase polymerase chain reaction results. These findings suggest that environmental stimulation can induce the expression of autoantigens such as SS-A/Ro. Key words: autoimmune disease; endocrine disruptors; environmental estrogens; RT-PCR; SS-A/Ro.**

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SS-A/Ro autoantibodies are often found in the sera of patients with systemic lupus erythematosus (SLE), subacute cutaneous lupus, neonatal lupus erythematosus, and the Sjögren syndrome (1–3). Because SLE is more common in women, some sex-related factors may be involved in the pathogenesis of these autoimmune diseases and also in the production of autoantibodies. Wang & Chan (4) recently demonstrated that the female sex hormone 17β-estradiol can induce cell surface expression of SS-A/Ro antigens in cultured normal human epidermal keratinocytes (NHEKs). Although this endogenous sex hormone is a steroid, a variety of exogenous non-steroids have been found to act like sex hormones such as 17β-estradiol. In fact, estrogenic xenobiotics (i.e. environmental estrogens), which are one class of endocrine-disrupting chemicals, have been implicated in a number of human health disorders (5–7). Estrogenic xenobiotics consist of naturally occurring compounds or commercially produced chemicals that mimic the action of 17β-estradiol. These substances are found in a number of relatively common and abundant sources such as pesticides, plastics, combustion by-products, plants, and agricultural products (8). Therefore, if the production of autoantibodies is governed at least in part by an antigen-driven process, the resulting linkage of the SS-A/Ro antigen expression to estrogenic stimulation may help explain the high frequency of anti-SS-A/Ro autoantibodies observed in

autoimmune diseases that affect predominantly women. To address this issue, *in vitro* experiments were designed to examine the expression of SS-A/Ro antigen in response to estrogenic xenobiotic treatment.

### MATERIALS AND METHODS

#### Chemicals

17β-estradiol, bisphenol-methoxychlor (insecticide), α-zearalanol (livestock anabolic), *p*-octylphenol (detergent), progesterone, and cholesterol were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Coumestrol (plant) was obtained from Acros Organics (Geel, Belgium). All reagents were of analytical grade.

#### Cell cultures

NHEKs from adult female breast skin without any specific disease were obtained commercially (Iwaki, Tokyo, Japan). They were maintained in phenol red-free keratinocyte growth medium (KGM) (Iwaki) (9) because phenol red has estrogenic activity (10). To examine the effects of 17β-estradiol and estrogenic xenobiotics on SS-A/Ro mRNA expression, the cells were cultured in KGM alone for 48 h and then cultured for an additional 72 h in KGM supplemented with one of several phenolic chemicals: 17β-estradiol (10 pM or 1.0 nM), coumestrol (1.0 µM or 10 µM), bisphenol-methoxychlor (1.0 µM or 10 µM), *p*-octylphenol (1.0 µM or 10 µM), or α-zearalanol (1.0 µM or 10 µM). Progesterone (1.0 nM or 10 nM) and cholesterol (1.0 µM or 10 µM) were also used as non-phenolic control substances. The dose ranges were determined as described previously (11).

#### Reverse transcriptase polymerase chain reaction (RT-PCR)

NHEKs were harvested from culture flasks using trypsin, and total cellular RNA was purified using UltraspecRNA isolation reagents (Biotex, Houston, TX, USA). SS-A/Ro antigen-specific RNA was analysed using a simplified RT-PCR design described by Wang & Chan (4). Total RNA (0.1–1.0 µg) and 0.5 µM primers (0.5 µl each) were heated at 70°C for 10 min and then quickly chilled on ice. The remaining components – including 1.25 U of *Taq* polymerase (Gibco BRL, Gaithersburg, MD, USA), 100 U of SuperScript II RNase H-reverse transcriptase (Gibco BRL), 20 U of RNase inhibitor (Toyobo, Tokyo, Japan), and 0.25 µl of 10 mM dNTPs; 2.5 µl of 10 × PCR buffer containing 500 mM KCl, 100 mM Tris-HCl, pH 8.3, 14 mM MgCl<sub>2</sub>, and 0.1% gelatin – were added to a final total reaction volume of 25 µl. The sense primer 5'-AAGCTCCAGGTGGCATTAG-3' and anti-sense primer 5'-CAGAGTTCATGGGGAAAAAGA-3' were used for detection of the 52-kDa SS-A/Ro mRNA, yielding the expected 1099 and 868 bp PCR products for 52α and 52β, respectively (12). All reaction components were mixed in a single 500 µl microfuge tube before thermal cycling. The RT-PCR programme consisted of a reverse transcription step (50°C for 1 h) and a denaturing step (94°C for 3 min), followed by 30 cycles of PCR (94°C for 5 sec, 55°C for 5 sec, and 72°C for 1 min). RT-PCR products were analysed by agarose gel electrophoresis. After the run was stopped, bands on the gel were visualized by ethidium bromide, and finally analysed in a scanning image-UV densitometer (BioRad Japan, Tokyo, JAPAN) for quantitative analysis.

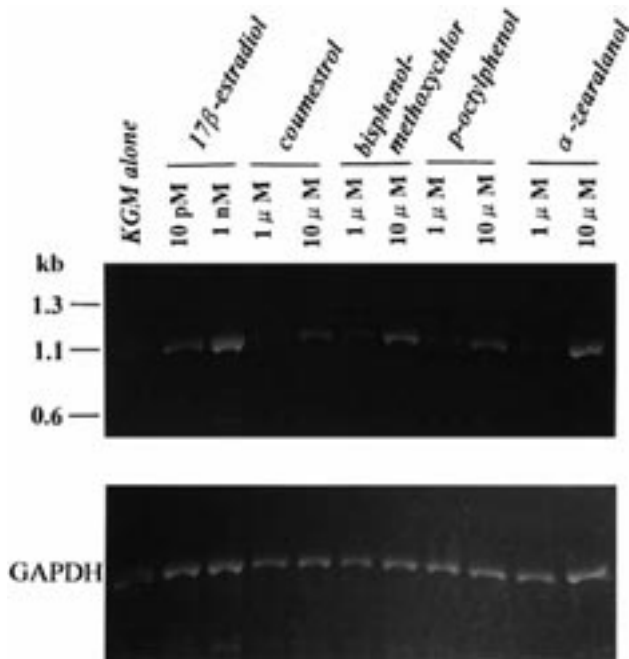


Fig. 1. (Upper panel) Increase in the level of 52-kDa (52 $\alpha$ ) SS-A/Ro autoantigen mRNA in normal human epidermal keratinocytes incubated with 17 $\beta$ -estradiol (10 pM or 1.0 nM) and various estrogenic xenobiotics (1.0  $\mu$ M or 10  $\mu$ M) for 3 days. Total RNA samples of 0.5  $\mu$ g each were analysed by RT-PCR. (Lower panel) No change in signal was detected for GAPDH mRNA using a 50-fold lower amount of total RNA. (KGM=keratinocyte growth medium, GAPDH=glyceraldehyde 3-phosphate dehydrogenase.)

#### Immunohistochemistry

To immunohistochemically stain SS-A/Ro protein molecules, NHEKs were fixed in 4% paraformaldehyde/phosphate-buffered saline (pH 7.4) at 25°C for 20 min. The fixed cells were then treated in the following solutions: (i) dilute normal goat serum as a protein blocking agent for 15 min; (ii) mouse monoclonal anti-52-kDa SS-A/Ro antibody (1:40) (Protein Biotechnik GmbH, Mannheim, Germany) for 17 h at 4°C; (iii) biotinylated rabbit anti-mouse IgG (Nichirei, Tokyo, Japan) for 12 h at 4°C, and (iv) streptavidin-alkaline phosphatase complex (Nichirei) for 1 h at 25°C. Binding was visualized using a revealing reagent at 25°C for 1 h in the dark.

## RESULTS

#### Effect of 17 $\beta$ -estradiol and estrogenic xenobiotics on the expression of SS-A/Ro mRNA

Fig. 1 shows the response of SS-A/Ro mRNA expression in NHEKs stimulated with different concentrations of 17 $\beta$ -estradiol and estrogenic xenobiotics. The full-length 52-kDa SS-A/Ro mRNA was detected as a 1.1-kb band. The alternative splice forms of 52 $\beta$  were not observed (data not shown). The 52-kDa SS-A/Ro mRNA level increased after treatment with 10 pM and 1.0 nM 17 $\beta$ -estradiol and 10  $\mu$ M estrogenic xenobiotics. However, at a concentration of 1.0  $\mu$ M, estrogenic xenobiotics had no effect on 52-kDa SS-A/Ro mRNA expression compared with untreated cells. No concentration of progesterone and cholesterol had a significant effect on SS-A/Ro mRNA expression (data not shown). The percentage induction of SS-A/Ro mRNA expression is shown in Fig. 2. The per-

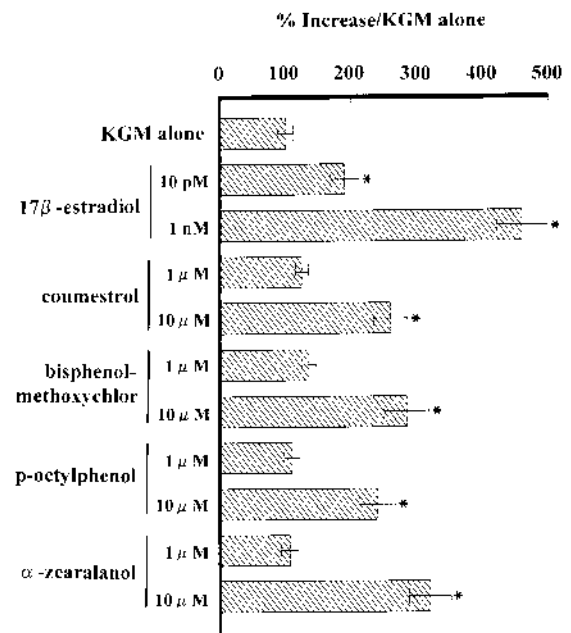


Fig. 2. Image analysis of the expression of 52-kDa (52 $\alpha$ ) SS-A/Ro mRNA in normal human epidermal keratinocytes. The photometric intensity at keratinocyte growth medium alone is designated as the "control value". Values are mean  $\pm$  SD. \* $p$  < 0.002 vs KGM alone control.

tage induction by all chemicals was significant when compared with SS-A/Ro mRNA expression in KGM alone. Although the mRNA of the housekeeping gene coding for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as an internal control (10 ng total RNA/reaction), the levels for GAPDH were approximately the same in each sample (Fig. 1).

#### Effect of 17 $\beta$ -estradiol and estrogenic xenobiotics on the expression of SS-A/Ro protein molecule

Control 52-kDa SS-A/Ro protein (SS-A/Ro)-positive cells stained purplish-blue and tended to cluster with expression of SS-A/Ro localized exclusively in the cytoplasm (Fig. 3a). Addition of 17 $\beta$ -estradiol to the culture medium brought about a gradual increase in intensity of SS-A/Ro-positive staining at doses over 10 pM (Fig. 3b, c). Addition of estrogenic xenobiotics increased the intensity of SS-A/Ro-positive staining at doses over 10  $\mu$ M. Fig. 3d shows the effect of bisphenol-methoxychlor on SS-A/Ro expression. When normal mouse serum was added to the slides instead of primary antibody, no SS-A/Ro-positive cells were detected (data not shown).

## DISCUSSION

The consequences of human exposure to environmental xenobiotics (i.e. endocrine disruptors) on reproductive function have been well studied (6–8, 13). However, little is known about the pharmacological and/or toxicological effects of such exposure on non-reproductive tissues, especially with respect to human health disorders such as autoimmune diseases. To address this issue, we focused this study on whether estrogenic xenobiotics promote autoantigen expression in a manner simi-

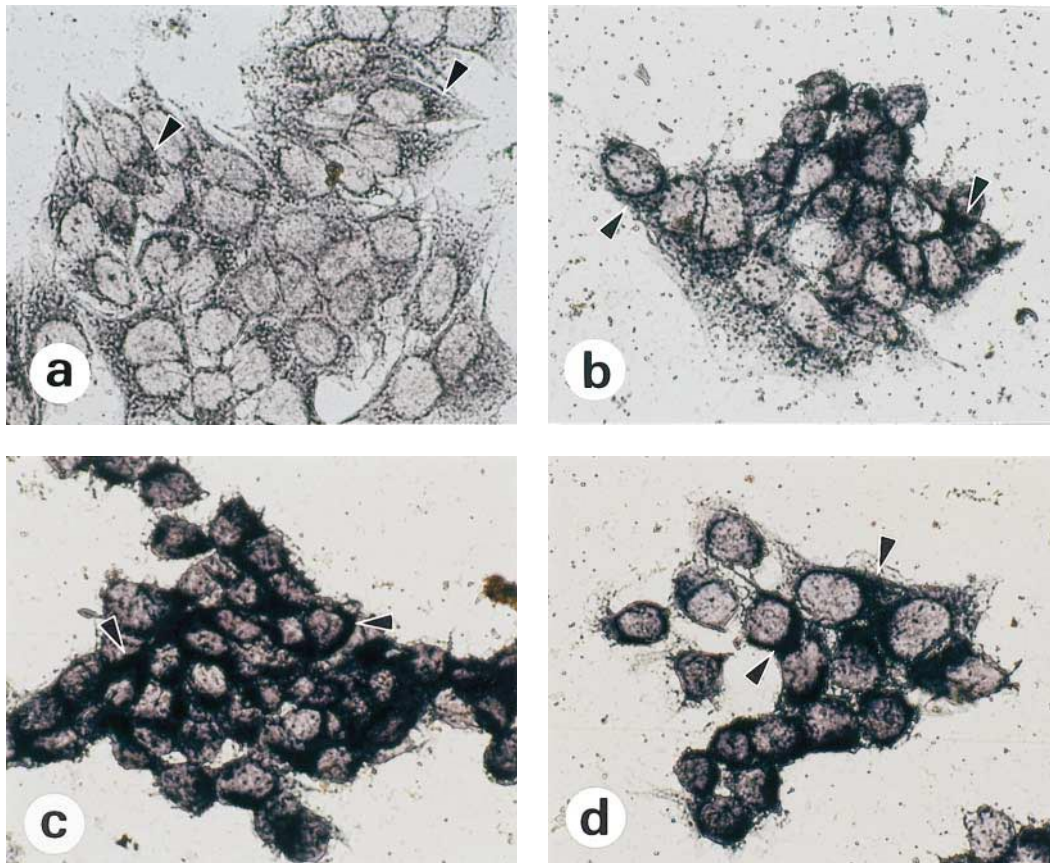


Fig. 3a–d. Immunohistochemical staining of SS-A/Ro in normal human epidermal keratinocytes. (a) Keratinocyte growth medium alone control. The SS-A/Ro-positive cells tend to cluster, and expression of SS-A/Ro is localized preferentially in the cytoplasm; (b) 10 pM  $17\beta$ -estradiol-treated cells; and (c) 1.0 nM  $17\beta$ -estradiol treated cells. The staining intensity has increased significantly. (d) 10  $\mu$ M bisphenol-methoxychlor-treated cells. The change in staining intensity of SS-A/Ro is seen as compared to the control ( $\times 360$ ). (Arrowhead) Cytoplasmic staining of SS-A/Ro.

lar to endogenous estrogen. This focus builds upon the previous finding (4) that estrogen may promote the appearance of SS-A/Ro autoantigen, which appears to have some association with the incidence of autoimmune disease. For example, Stoeber et al. (14) reported tamoxifen, an estrogen antagonist, to have a beneficial therapeutic effect on the development and course of murine experimental SLE. In 1988, Furukawa et al. (15) showed enhanced binding of anti-SS-A/Ro autoantibody to cultured NHEKs treated with  $17\beta$ -estradiol.

In this study, we used RT-PCR and immunohistochemistry to examine the effects of natural estrogen (i.e.  $17\beta$ -estradiol) and various estrogenic xenobiotics on expression of the 52 kDa SS-A/Ro autoantigen in NHEKs. The results indicate that natural estrogen and estrogenic xenobiotics promote the appearance of SS-A/Ro antigen at both the mRNA and protein levels, and that estrogenic xenobiotics derived from plants, insecticides, detergents, or livestock anabolics have this action, although to varying degrees. Hormonal depletion of the human epidermal cells led to decreased levels of SS-A/Ro mRNA and protein, and the addition of natural estrogen and estrogenic xenobiotics led to an increase in SS-A/Ro expression. The effect was seen at 10  $\mu$ M levels, which are rarely present in the environment. Thus, these estrogenic xenobiotics cannot be regarded as risk factors for autoimmune disease under ordinary conditions or ordinary living conditions; however, Olea & Olea-Serrano (16) recently demonstrated that estro-

genic xenobiotics were present in both extracted foods and water from autoclaved cans at concentrations of mg level per can, for example. Thus, the results of the present study suggest that we must recognize the possibility that estrogenic xenobiotics can induce the expression of autoantigens such as SS-A/Ro.

Although, the precise mechanism by which estrogenic xenobiotics affect SS-A/Ro expression is still unclear, Urano et al. (17) showed the presence of estrogen receptors and mRNA in NHEKs. Furthermore, Wang & Chang (4) recently reported on a putative estrogen response element in the human gene encoding 52 kDa SS-A/Ro. Considering these findings, it is clear that the present estrogenic effect was mediated through the estrogen receptor. Further efforts to resolve this question are under way in our laboratory.

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