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Novel effect of estrogen on RANK and *c-fms* expression in RAW 264.7 cells

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Abstract. Temporomandibular disorder (TMD), a progressive disease entity, and osteoarthrosis preferentially affect females, denoting a possible role of estrogen. Using RAW 264.7 cells, the expression of estrogen receptors (ERs) α and ß and the consequent effect of estrogen was investigated. We present the novel detection of ER ß expression in RAW 264.7 cells. Furthermore, we innovatively demonstrated the increase in expression of both ER α and ß, as well as RANK and *c-fms*, with estrogen treatment. However, a decrease in expression of *c-fms*, RANK and ER ß, and nearly no change in the expression of ER α were experienced upon further increase in estrogen concentrations. These findings lead us to hypothesize a new mechanism of inflammation in TMD.

Introduction

Estrogen, a representative steroid hormone, is known to regulate diverse physiological processes of target tissues (1) in both sexes. The biological activities of estrogen are initiated by binding to the specific receptor proteins, namely the estrogen receptors (ERs) (2). Two main isoforms of ER have been identified to date, ER α and ER ß. The latest discovery of ER ß was unexpected during a search for novel androgen receptors in a rat prostate cDNA library (3). Immunocytochemistry using specific antibodies revealed that ER α was widely distributed in various tissues, whereas the definitive distribution of ER ß protein remained unclear (2).

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The nuclear receptor (NR) genes are a large family of genes, coding for similar proteins, whose functions are to accept incoming signals from various messenger molecules. NR genes are typically found on the nuclear membrane or less frequently, free in the cell cytoplasm (4). The steroid nuclear receptors are a sub-family of the NR super-family, mediating the cellular effects of steroid hormones. Estrogen is a key regulator of skeletal mass. The effects of estrogen on osteoblasts include both direct effects, resulting in increased bone formation, and indirect effects, via an osteoblast-mediated interaction with pre-osteoclasts and osteoclasts, resulting in decreased bone resorption (5). The effects of estrogen on skeletal cells are complex, and the mechanism(s) of action are controversial (6).

RANKL (receptor activator of NFκB ligand) is a membrane-bound ligand expressed on osteoblasts. Binding of RANKL to its receptor RANK which is expressed on osteoclast precursors and mature osteoclasts, induces osteoclastogenesis and activation of mature osteoclasts (7-11). RANK is a member of the TNF-R super-family, expressed as a transmembrane heterotrimer on the surface of haematopoietic osteoclast progenitors, mature osteoclasts, chondrocytes, and mammary gland epithelial cells (12-16). Estrogen has been shown to suppress RANKL-induced osteoclast differentiation *in vitro* (17). Macrophage colony stimulating factor-1 (M-CSF, CSF-1) is essential for osteoclastogenesis, as demonstrated by osteopetrotic defects in both M-CSF and M-CSF receptor (*c-fms*) knockout mice (18,19), and for the survival of mature osteoclasts (20). Importantly, M-CSF stimulates the spreading and migration of osteoclasts together with the attenuation of bone resorption (21). The *c-fms* gene encodes the receptor for macrophage colony-stimulating factor-1. This gene is expressed selectively in the macrophage cell lineage (22).

The synovial membrane in the temporomandibular joint (TMJ) has an important role in joint movement because of the involvement of the synovial lining cells in the synovial fluid metabolism, which affects smooth jaw movement. Many ultrastructural investigations have pointed out that the synovial membrane consists of two kinds of synovial lining cells; macrophage-like type A and fibroblastic type B cells (23,24). Taken together, these findings lead us to hypothesize that the

TMJ is a target tissue for estrogen. However, little information is available regarding the localization of ER α and ß in the TMJ.

Available precedents suggest that the effects of estrogen on osteoclast progenitors and osteoclast differentiation may be more important than the effects on formed osteoclasts (25-27). Our study targeted the effects of estrogen on osteoclast precursors represented by the murine monocytic line RAW 264.7. We detected the presence of ERs in RAW 264.7 cells and examined the effects of estrogen on RAW 264.7 cells regarding the *c-fms* and RANK receptors.

Materials and methods

Cell culture. RAW 264.7 cells were first cultured in complete medium α-MEM supplemented with 66.7% μg/ml kanamycin and 10% fetal calf serum (FCS). Cells were incubated in a humidified 5% CO_2 atmosphere at 37˚C.

Exogenous 17ß-estradiol treatment. At confluence, different concentrations (10^{-8}, 10^{-9}, 10^{-10} and 10^{-11} M) of 17ß-estradiol (in ethanol, Sigma-Aldrich, St. Louis, MO) were added to the medium for periods of 1, 3, 6, 12 and 24 h, while controls were treated with an equivalent volume of ethanol (estrogen vehicle). Exposure was terminated by rapid aspiration of the media.

Reverse transcription (RT)-polymerase chain reaction (PCR). Total RNA was isolated using guanidinium thiocyanate-phenol-chloroform extraction (TRIzol, Invitrogen Corp., Carlsbad, CA). The RNA pellets were dissolved in distilled water and quantified using a Nano-Drop spectrophotometer ND-1000 (NanoDrop Tech., Inc., Wilmington, DE). Total RNA was reverse transcribed using oligo(dt) primers and reverse transcriptase (Toyobo, Tokyo, Japan) according to the manufacturer's protocol.

PCR amplification. For expression of ER α and ß, a mixture of 0.16 mM dNTP, 0.4 μM primer F, 0.4 μM primer R, and KOD-Dash (Toyobo) was prepared. At first, gene-specific primers for human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were used to test cDNA quality. Amplification was performed as follows: 35 cycles at 94˚C for 30 sec, annealing at 60˚C for 2 sec, and 72˚C for 30 sec. The first cycle was conducted at 94˚C for 10 min and the final cycle at 72˚C for 10 min.

For *c-fms* and RANK a mixture of 0.16 mM dNTP mix, 1 μl of each sense and antisense primer (0.4 μM), and Tag gold was used. Gene-specific primers for ß-2 microglobulin were used to test cDNA quality and equalization. Amplification was performed as follows: 30 cycles with the first cycle conducted at 95˚C for 11 min and at 95˚C for 1 min, with annealing at 58˚C and 72˚C for 1 min, and with the last cycle at 72˚C for 10 min. The sequences of the used primers are as shown in Table I.

Following PCR, the reaction products were resolved on 2% agarose gel by electrophoresis. Gel was stained with ethidium bromide to visualize the PCR products. The levels of expression of ER α, ER ß, RANK and *c-fms* were analyzed with Image J 1.37 v (Rasband, W.S., ImageJ, US National Institute of Health, Bethesda, MD, USA, 1997-2006) and normalized with ß-2 microglobulin mRNA expression.

Table I. The sequences of the used primers.

Primer	Sequence
ER α	
Forward	CAAGGAGGGAGTGCGTCTGG
Reverse	CATCTAGGACCAGGTCCTCAGC
ER ß	
Forward	TCTGCAGTGATTATGCATCTGGG
Reverse	CTTCGTGAGGGACATCATCATGG
RANK	
Forward	CCA GGG GAC AAC GGA ATC A
Reverse	GGC CGG TCC GTG TAC TCA TC
c-fms	
Forward	GCG ATG TGT GAG CAA TGG CAG T
Reverse	AGA CCG TTT TGC GTA AGA CCT G
G3PDH	
Forward	GTCTACATGTTCCAGTATGACTCC
Reverse	AGCCTTCTCCATGGTGGTGAAGAC
ß-2 microglobulin	
Forward	GCT ATC CAG AAA ACC CCT CAA
Reverse	CATGTCTCGATCCCAGTAGACGGT

Western blotting. For protein extraction, at confluence, media were aspirated, cells were washed with Dulbecco's phosphate-buffered saline without calcium and magnesium (1X PBS), and lysis buffer was added (10 mM HEPES-KOH at pH 7.5, 100 mM KCl, and 0.1% NP-40). Cells were lysed thoroughly and dislodged from the plate by repetitive pipetting. Protein concentrations were assessed by the Bio-Rad protein assay kit. Controls for both α and ß were used (MCF7 whole cell lysate for α and NIH/3T3 whole cell lysate for ß, Santa Cruz Biotechnology, Inc., CA). Samples were separated on 10% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Membranes were blocked for 1 h in 5% skim milk followed by 3 washings with TBS containing 0.1% Tween buffer and were placed in a primary antibody [1:200 ER α (H-184) rabbit polyclonal IgG and 1:500 ER ß rabbit polyclonal IgG, Santa Cruz Biotechnology, Inc.] overnight at 4˚C on a shaker, followed by 3 washings with TBST and placed in a secondary antibody (1:2500 HRP-goat anti-rabbit IgG, Zymed, San Francisco, CA) for 1 h at room temperature on a shaker. After 3 washings for 15 min, the membranes were incubated in Western Lightning® Chemiluminescence Reagent Plus (Perkin Elmer, Boston, MA). Medical x-ray films were used to visualize the chemiluminescence.

Results

Expression of estrogen receptors in RAW 264.7 cells. ERs are central to the study, so we examined the expression of ER α and ß in RAW 264.7 cells. Using PCR (a 35-cycle amplification) both ER α and ß were expressed as a specific single band in RAW 264.7 cells (Fig. 1). On the other hand, Western

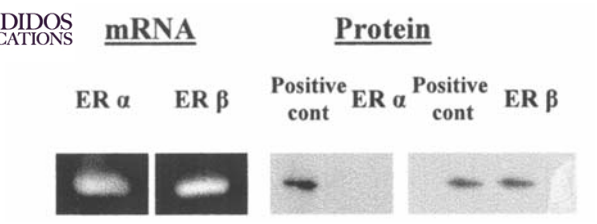

Figure 1. (Left panels) Estrogen receptors ER α and ß RNA expression in macrophage cells. RAW 264.7 cells were analyzed by RT-PCR for the presence of the mRNA encoding ER α and ß. Cells were cultured under standard growth conditions, and RNA was extracted and retrotranscribed using the reverse transcriptase enzyme. ER α and ß were expressed as specific single bands. (Right panels) Western blot analysis for ERs. The whole cell lysates from RAW 264.7 cells were analyzed for the expression of ER α and ß. The positive controls were MCF7 whole cell lysate for α and NIH/3T3 whole cell lysate for ß. Cont, control.

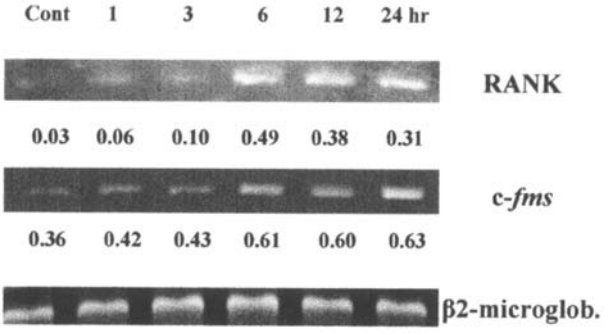

Figure 2. The effect of 17ß-estradiol on RANK and *c-fms* mRNA expression in RAW 264.7 cells. Cells were incubated with 10^{-11} M 17ß-estradiol for periods of 1, 3, 6, 12 and 24 h. RNA was isolated and RT-PCR was performed. There was a gradual increase in expression of both RANK and *c-fms* after 1 h, which then obviously increased at 6 h. Cont, control.

blotting confirmed the expression of ER ß, while no adequate signal was detected for ER α (Fig. 1).

A band corresponding to the expected size of G3PDH was detected (data not shown).

Effect of estrogen on RAW 264.7 cells. At confluence, following the addition of 10^{-11} M 17ß-estradiol (in ethanol) to the medium for periods of 1, 3, 6, 12 and 24 h, the effect of estrogen on RANK and *c-fms* in RAW 264.7 cells was investigated. PCR amplification for 30 cycles revealed that estrogen directly affected expression of RANK and *c-fms*. cDNA quality and equality were checked by ß-2 micro-globulin. For both RANK and *c-fms* there was increased expression after 1 h following the addition of estrogen. There was a time-dependent increase in the expression of RANK and *c-fms* after application of estrogen with maximum expression at 6 h (Fig. 2).

Effect of estrogen on ER receptors. The capacity of estrogen to increase expression of RANK and *c-fms* and the detection of ERs in RAW cells suggested mediation by ERs (ER α or ER ß) so we studied the effect of estrogen on ERs. The expression of both ER α and ß after the addition of 10^{-11} M

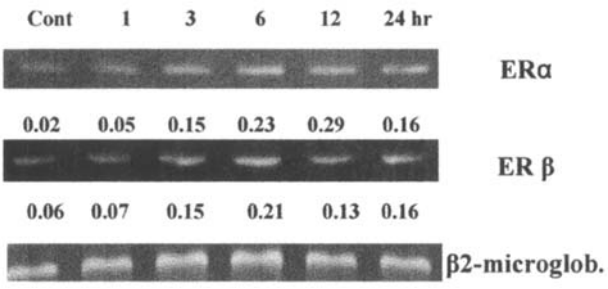

Figure 3. The effects of 17ß-estradiol on ER α and ß mRNA expression in RAW 264.7 cells. Cells were incubated with 10^{-11} M 17ß-estradiol for periods of 1, 3, 6, 12 and 24 h. RNA was isolated, and RT-PCR was performed. A gradual increase in expression of ER α and ß with time was observed, with the obvious effect taking place after 6 h. Cont, control.

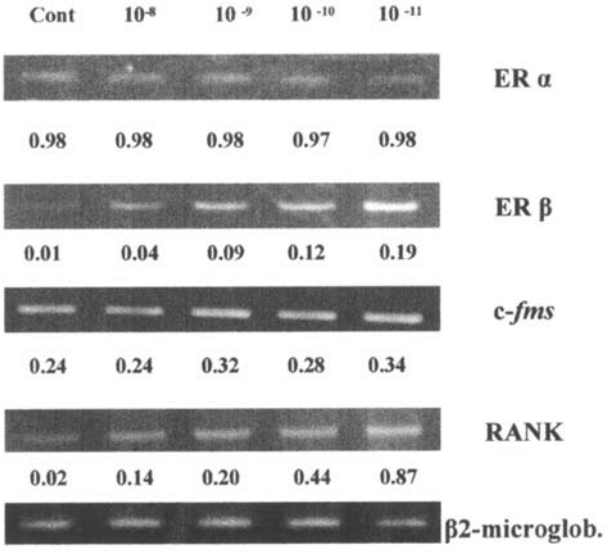

Figure 4. The effect of different concentrations of 17ß-estradiol on the expression of *c-fms*, RANK and ERs. PCR amplification was conducted to study the effect of different concentrations on the expression of RANK, *c-fms* and ERs. There was nearly no change in the expression of ER α at different concentrations of 17ß-estradiol, while there was a gradual decrease in expression of RANK and *c-fms* with increasing estrogen concentration, with a similar decrease in ER ß expression. Cont, control.

17ß-estradiol for periods of 1, 3, 6, 12 and 24 h was studied. PCR amplification of 30 cycles was conducted for both ERs. There was a gradual increase in expression of both ER α and ß with time, with the obvious effect taking place after 6 h and a slight decrease in expression of ER α at 24 h (Fig. 3).

Concentration dependency of estrogen. At confluence, different concentrations (10^{-8}, 10^{-9}, 10^{-10} and 10^{-11} M) of 17ß-estradiol (in ethanol) were added to the medium for a period of 6 h. PCR amplification was performed to study the effect of different concentrations on the expression of RANK, *c-fms* and ERs. There was nearly no change in the expression of ER α at concentrations 10^{-11} to 10^{-8} M, while there was a gradual decrease in expression of RANK and *c-fms* with increase in estrogen concentration, with the same decrease occurring in ER ß expression (Fig. 4).

Discussion

As direct targets for pharmaceuticals, estrogen receptors represent a promising biological system for the discovery of potent, selective and specific drugs to control the evolution of several disorders (28). Macrophage cell lines expressing endogenous estrogen receptors represent valuable tools in the study of hormonal activity on inflammatory cells and to discern the mechanism of action of hormone and hormone-related drugs.

Previous studies have failed to detect ER ß in RAW 264.7 cells (29), but in this study we present its novel detection in RAW 264.7 cells. We have also demonstrated the expression of ER α receptor. Results from our present study demonstrated increased expression of both ER α and ß in RAW 264.7 cells with time after application of 10^{-11} M estrogen. Since the synovial membrane consists of two kinds of synovial lining cells; macrophage-like type A and fibroblastic type B cells (23,24), this proves that type A synovial lining cells possess estrogen receptors through which estrogen can exert its effect on the joint.

Macrophages are the patrolling cells of innate immunity, involved in the recognition of foreign pathogens, in the elimination of toxic molecules and in the reconstitution of tissue integrity. Macrophage cells orchestrate these diverse pathways by producing several different mediators, such as nitric oxide (NO), cytokines including interleukin 1ß (IL-1ß) and tumor necrosis factor-α (TNF-α), and matrix degrading enzymes such as metalloprotease-9 (MMP-9). The production of these molecules, although beneficial for killing bacteria and further activating the immune system, exerts damaging effects on the surrounding tissue when chronically or erroneously stimulated (30).

The differentiation of macrophages from bone marrow progenitors requires the coordinated expression of many genes needed for mature cell function. This process is controlled by the lineage-specific growth factor macrophage colony-stimulating factor-1 (CSF-1), which acts by binding to cell surface receptors (CSF-1Rs) encoded by the *c-fms* proto-oncogene (31). Our results revealed an increased expression of *c-fms* following estrogen treatment, based on which we hypothesized a resultant increase in macrophage colonization via *c-fms*. Such a hypothesis, in an attempt to scientifically explain a female tendency to TMD, might interpret the increase in macrophage colonization to be a contributing factor in the increased inflammation and tendency of females to suffer more from temporomandibular joint disorders in the presence of external exacerbating factors.

Until recently, inflammatory cytokines, such as IL-1, IL-6, IL-11, and TNF, were implicated as important mediators of bone lysis (32). Recent findings, however, indicate that osteoclasts have a substantial role in bone destruction, and that RANKL might be the central mediator of osteoclast development.

In the presence of CSF-1 sufficient to maintain cell growth and survival, RANKL, via its tumor necrosis factor family receptor RANK, is sufficient to induce complete osteoclastic differentiation from hematopoietic precursors (33). It has been established that estrogen withdrawal causes rapid skeletal degradation, an effect that certainly involves other cells, including osteoblasts, but which has been hypothesized to involve direct effects on osteoclast formation (34).

Previous studies reported that estrogen does not affect RANK (17). However, our current study substantiates a direct effect of estrogen on RANK in osteoclast precursors where addition of 17ß-estradiol caused a direct increase in RANK expression. We hypothesize that the increase in expression of RANK is a reaction of the RAW 264.7 cells (osteoclast precursors) to compensate for the decreased level of RANKL caused by estrogen (17).

Estrogens have been demonstrated to act via ER ß in tissues of the central nervous system, cardiovascular system, immune system, urogenital tract, gastrointestinal tract, kidney and lungs (35). We demonstrated that 10^{-11} M of estrogen is the optimum concentration for estrogen effects via ER ß, and that higher concentrations caused a decreased expression of ER ß. Higher estrogen concentrations beyond optimal also caused a contemporaneous decrease in RANK and *c-fms* while having no effect on the expression of ER α, denoting a coexisting relation between estrogen and the ER ß receptor.

In conclusion, we report that type A synovial lining cells express ERs α and ß which are increased by estrogen treatment and we hypothesized an increase in macrophage colonization via the reported increase in *c-fms*. Furthermore, we demonstrated that osteoclast precursors are direct targets of estrogenic effects. These results may provide an interpretation for the increased inflammation and tendency of females to suffer from temporomandibular joint disorders; hence, providing novel targets for both prevention and therapy.

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