ORIGINAL

Effects of raloxifene on the production of cytokines in stimulated whole blood in *ex vivo* and *in vitro* studies

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Anna Tani[b], Sumika Matsui[b], Akira Kuwahara[b], Toshiya Matsuzaki[b],
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Abstract : Purpose : The aims of this study were to determine the effects of raloxifene therapy on production of cytokines and *in vitro* effects of raloxifene on production of cytokines by whole blood cultures. Methods : We obtained samples of peripheral blood from 6 postmenopausal women with osteopenia at baseline and after 3 and 6 months of raloxifene therapy and 10 postmenopausal women who did not receive raloxifene therapy. Whole blood from raloxifene-treated women was stimulated with lipopolysaccharide (LPS) or phytohemeagglutinin (PHA). Whole blood from postmenopausal women who were not treated with raloxifene was preincubated with raloxifene at concentrations of 10^{-10}-10^{-7} M and then stimulated with LPS or PHA. Concentrations of IL-1β, IL-4, IL-6, IL-12p40, IL-12p70, TNF-α and IFN-γ in the supernatant were measured by respective ELISAs. Results : In *ex vivo* cultures, raloxifene therapy inhibited LPS-stimulated production of IL-1β, IL-6, IL-12p40, IL-12p70 and TNF-α, but not PHA-stimulated production of IL-4 and IFN-γ. In *in vitro* cultures, raloxifene at a concentration (10^{-9} M) inhibited LPS-stimulated production of IL-1β, IL-6 and IL-12p40 and PHA-stimulated production of IFN-γ. Conclusions : Raloxifene therapy decreases the production of IL-1β, IL-6, IL-12 and TNF-α but not that of IL-4 and IFN-γ, suggesting that modulation of cytokines could play a role in the mechanisms of the osteoprotective effect of raloxifene. J. Med. Invest. 58 : 110-117, February, 2011

Keywords : *raloxifene, cytokine, whole blood assay*

INTRODUCTION

Dramatic changes in the endocrine system occur

in women at menopause. The decline in estrogen production results in various degenerative conditions, such as osteoporosis and atherosclerosis. Hormone replacement therapy (HRT) is widely used to prevent or delay the occurrence of these disorders in postmenopausal women. Estrogen modulates the production of cytokines since immunocompetent cells possess estrogen receptors (1, 2).

Inflammatory cytokines such as interleukin-1

(IL-1), IL-6 and tumor necrosis factor-α (TNF-α) are known to be bone-resorbing cytokines based on many experimental models (3-5). There are many reports demonstrating that estrogen replacement decreases the production of IL-1, IL-6, IL-12 and TNF-α by peripheral blood mononuclear cells (PBMC) (6, 7) and whole blood cultures (8). We have also demonstrated that HRT inhibited the production of IL-1β, IL-4, IL-10, interferon-γ (IFN-γ) and TNF-α in whole blood cultures (9-11) since the whole blood assay is a potentially valuable tool for assessing cellular immune function (12, 13).

Raloxifene, a non-steroidal benzothiophene derivative classified as a selective estrogen receptor modulator (SERM), has been used for the treatment of postmenopausal osteoporosis. Although raloxifene binds with high affinity to the estrogen receptor, the conformation of the ligand/receptor complex for raloxifene and estrogen differs, resulting in the differences seen in the pharmacological properties of raloxifene and estrogen. Recently, raloxifene has been reported to have effects on circulating cytokines such as IL-6 and TNF-α, but the results of studies on changes in these cytokines caused by raloxifene were inconsistent (14-17). The effects of raloxifene on production of bone-resorbing cytokines were also discrepant because raloxifene treatment inhibited the production of IL-6 and TNF-α in whole blood cultures but not the production of IL-1β in PBMC in postmenopausal women (15, 18). Furthermore, the effects of raloxifene therapy on production of cytokines such as IL-4, IFN-γ and IL-12, which acts as a critical bridge between the innate and acquired immune systems, remain to be established.

The aims of this study were to determine the effects of raloxifene therapy for 3 months and 6 months on cytokines in whole blood cultures and to compare with *in vitro* effects of raloxifene on stimulated secretion of cytokines in whole blood cultures.

MATERIALS AND METHODS

Subjects

Sixteen women were enrolled in this study after they gave informed consent. This project had been approved by the Institutional Review Broad of the University of Tokushima, Institute of Health Biosciences. The subjects included 10 untreated postmenopausal women (ages, 50 to 65 years ; mean age, 56.6 years) and 6 untreated postmenopausal women with osteopenia (ages, 52 to 65 years ; mean age, 56.2 years) from the Outpatient Clinic of the Department of Obstetrics and Gynecology, Tokushima University Hospital. The subjects had no overt disease and their peripheral blood cell counts, differential leukocyte count, and results of liver and renal function tests were within normal limits.

The 6 postmenopausal women with osteopenia received oral administration of 60 mg raloxifene HCl (Evista, Chugai, Japan) every day. Blood samples for cytokine evaluation were drawn into tubes before and 3 to 6 months after the start of raloxifene therapy.

Reagents

Raloxifene (LY139481) was kindly provided by Lilly Research Laboratories (Indianapolis, IN, USA). We used the following reagents : RPMI 1640 (Gibco, Grand Island, NY, USA), lipopolysaccharide (LPS) from Esherichis coli 055 (Difco, Elkhart, IN, USA), phytohemeagglutinin (PHA) (Sigma, St Louis, Mo, USA), and Ficoll-Paque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Ex vivo study

Whole blood cultures were prepared using previously described methods (9, 10). In brief, 1 ml of peripheral blood was withdrawn into a heparinized (10 U/ml) tube, and within 1 h, 0.1 ml of heparinized blood was cultured in a 24-well multicluster plate in 1 ml RPMI 1640 containing 10% fetal calf serum with 1 μg/ml LPS or 10 μg/ml PHA at 37°C for 24 h. The culture supernatants were collected and stored at -40°C until assayed.

In vitro study

The production of cytokines by peripheral blood cells from postmenopausal women was examined by whole blood culture. One ml of peripheral blood was withdrawn into a heparinized (10 U/ml) tube, and within 1 h, 0.1 ml of heparinized blood was cultured in a 24-well multicluster plate in 1 ml RPMI 1640 alone (control) or with raloxifene at doses of 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} M at 37°C for 12 h. Then whole blood cultures were stimulated by the addition of 100 ng/ml LPS or 1 μg/ml PHA at 37°C for 24 h. The culture supernatants were collected and stored at -40°C until assayed.

Measurement of concentrations of cytokines

Concentrations of IL-1β, IL-4, IL-6, TNF-α and IFN-γ in the supernatants were measured using

the respective human ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. The sensitivity level of the kits was 4 pg/ml. The intra- and interassay coefficients of variation were less than 10%. The concentration of IL-12p40 was measured using a human ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The sensitivity level of the kit was 15 pg/ml. The intra- and inter-assay coefficients of variation were 4.3% and 9.3%, respectively. The concentration of IL-12p70 was measured using a human ELISA kit (BioSource Internationnal, Camarillo, CA, USA) according to the manufacturer's instructions. The sensitivity level of the kit was 0.5 pg/ml. The intra- and interassay coefficients of variation were 9.5% and 8.7%, respectively.

Assessment of cell viability

Viability of cells treated with raloxifene was determined by the trypan blue dye exclusion test. In brief, peripheral blood mononuclear cells (PBMC) were isolated by fractionation on Ficoll-Paque Plus. The interface was removed and washed twice in RPMI 1640. Isolated PBMC were incubated as a density of 5×10^5/ml in RPMI 1640 alone (control) or with different concentrations of raloxifene (10^{-10},

10^{-9}, 10^{-8} and 10^{-7} M) at 37°C for 36 h. After the incubation, cells were stained with 0.2% trypan blue for 15 min. The number of stained cells among 200 cells was counted.

Statistical analysis

Statistical analyses comparing treatment groups were performed by the Wilcoxon signed-rank test. Values of $p<0.05$ were considered to be significant. The StatView 4.1 program (Abacus Concepts, Inc., Berkeley, CA) was used for statistical analysis.

RESULTS

Ex vivo study

Raloxifene therapy for 3 and 6 months induced a significant decrease in LPS-stimulated production of IL-1β ($p<0.05$), IL-6 ($p<0.05$) and TNF-α ($p<0.05$) in postmenopausal women (Fig. 1). Raloxifene therapy for 3 and 6 months also induced a significant decrease in LPS-stimulated production of IL-12p40 ($p<0.05$) and IL-12p70 ($p<0.05$) (Fig. 2). However, there was no significant change in either IL-4 or IFN-γ after 3 and 6 months of raloxifene therapy (Fig. 3).

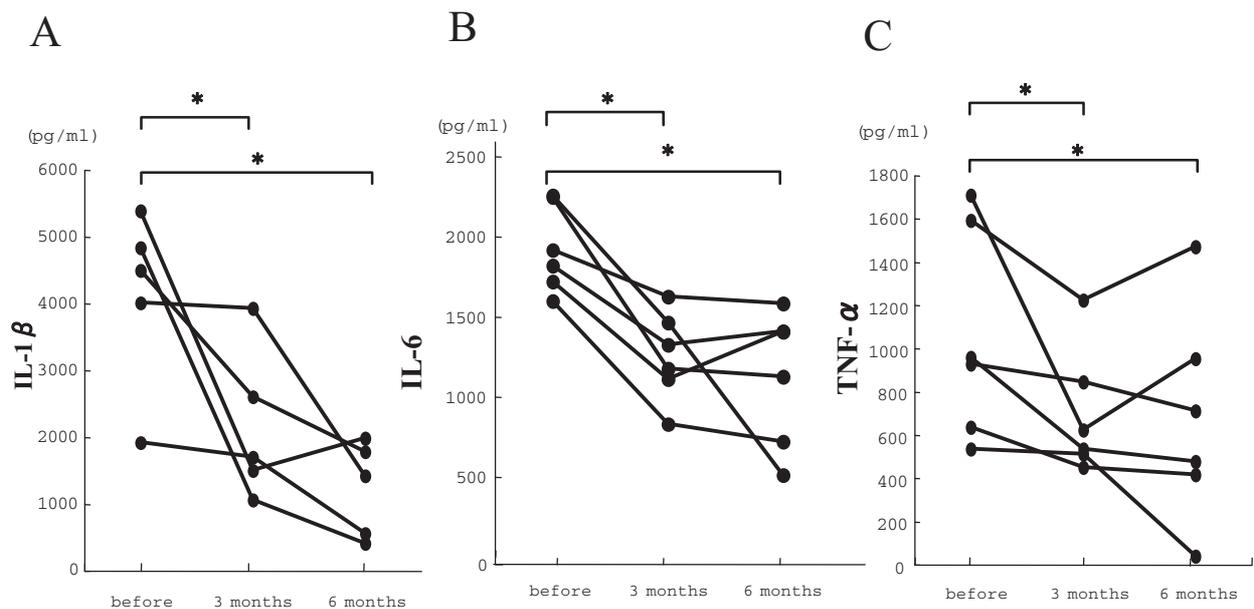Figure 1.   Effects of raloxifene therapy on production of IL-1β (A), IL-6 (B) and TNF-α (C) by 1 μg/ml LPS-stimulated whole blood cells from postmenopausal women with osteopenia. * indicates significance of difference from levels before therapy (p< 0.05).
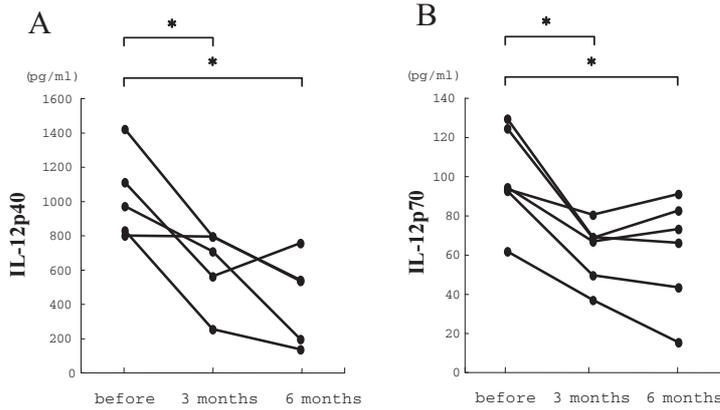
Figure 2.   Effects of raloxifene therapy on production of IL-12p40 (A) and IL-12p 70 (B) by 1 μg/ml LPS-stimulated whole blood cells from postmenopausal women with osteopenia. * indicates significance of difference from levels before therapy (p< 0.05).
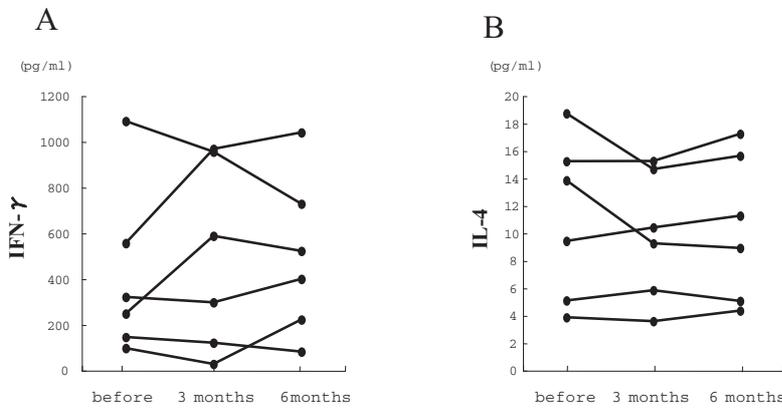

Figure 3.   Effects of raloxifene therapy on production of IL-4(A) and IFN-γ (B) by 10 μg/ml PHA-stimulated whole blood cells from postmenopausal women with osteopenia. * indicates significance of difference from levels before therapy (p< 0.05).

In vitro study

There was a significant decrease in IL-1β and IL-6 compared to basal levels at raloxifene doses of 10^{-9}, 10^{-8} and 10^{-7} M (p<0.05) (Fig. 4A, 4B). There was a significant decrease in TNF-α compared to basal levels at raloxifene doses of 10^{-8} and 10^{-7} M (p<0.05) (Fig. 4C). There was a significant decrease in IL-12p40 compared to basal levels at raloxifene doses of 10^{-9}, 10^{-8} and 10^{-7} M (p<0.05) (Fig. 5A), and there was a significant decrease in IL-12p70 compared to basal levels at a raloxifene dose of 10^{-10} and 10^{-8} M (p<0.05) (Fig. 5B).

There was a significant decrease in IFN-γ compared to basal levels at raloxifene doses of 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} M (p<0.05) (Fig. 6A), and there was a significant decrease in IL-4 compared to basal levels at raloxifene doses of 10^{-8} and 10^{-7} M (p<0.05) (Fig. 6B).

Cell viability

The viability of cells treated with raloxifene at doses of 10^{-10}, 10^{-9}, 10^{-8}
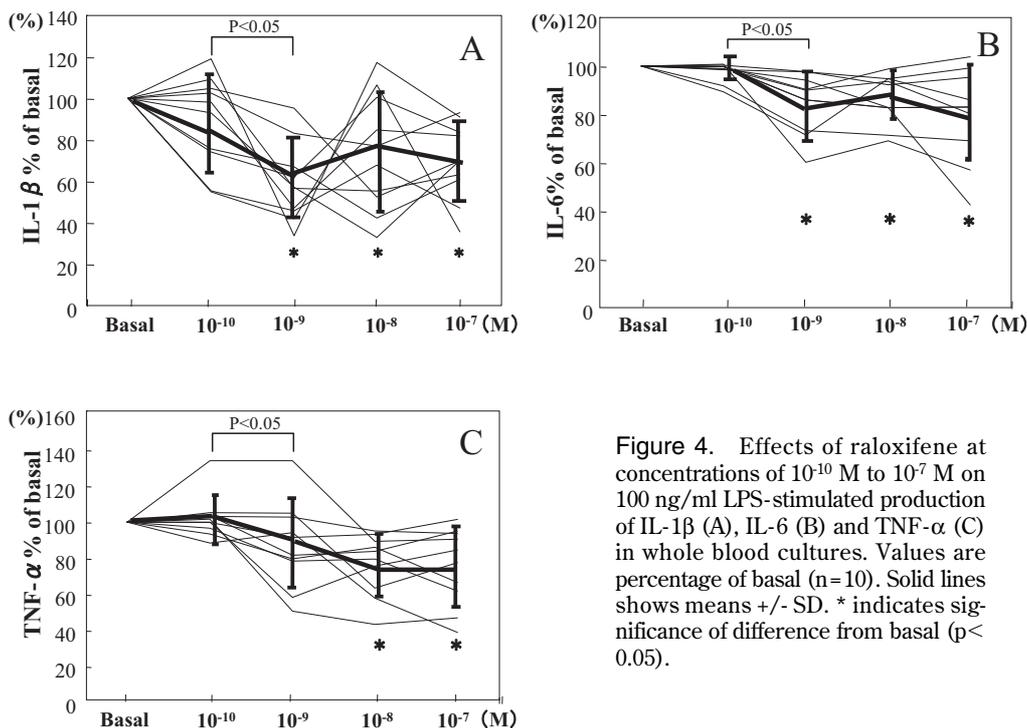

Figure 4.   Effects of raloxifene at concentrations of 10^{-10} M to 10^{-7} M on 100 ng/ml LPS-stimulated production of IL-1β (A), IL-6 (B) and TNF-α (C) in whole blood cultures. Values are percentage of basal (n=10). Solid lines shows means +/- SD. * indicates significance of difference from basal (p< 0.05).
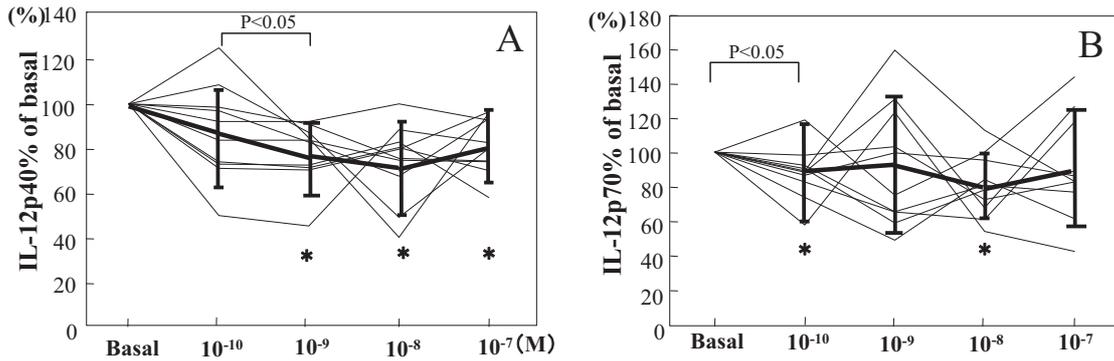
Figure 5. Effects of raloxifene at concentrations of 10^{-10} M to 10^{-7} M on 100 ng/ml LPS-stimulated production of IL-12p40 (A) and IL-12p70 (B) in whole blood cultures. Values are percentage of basal (n=10). Solid lines shows means +/- SD. * indicates significance of difference from basal (p<0.05).

Figure 6. Effects of raloxifene at concentrations of 10^{-10} M to 10^{-7} M on 1 μg/ml PHA-stimulated production of IL-4(A) and IFN-γ (B) in whole blood cultures. Values are percentage of basal (n=10). Solid lines shows means +/− SD. * indicates significance of difference from basal (p<0.05).
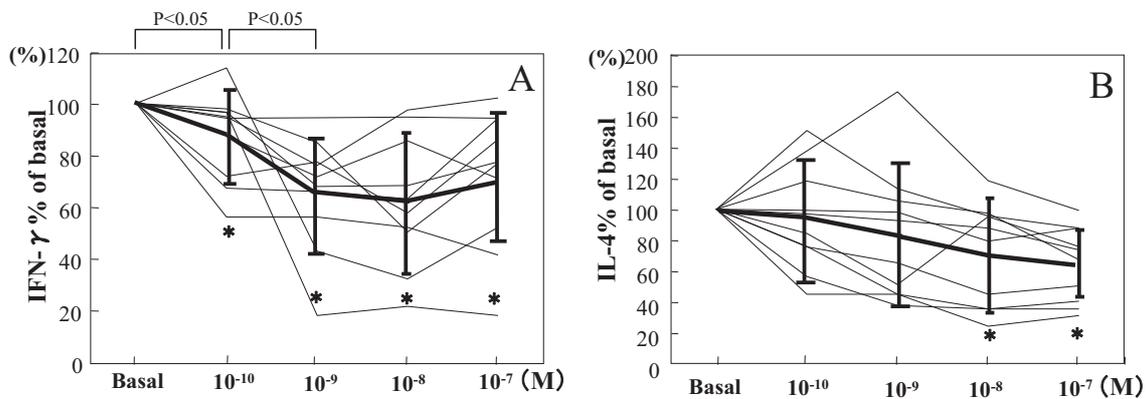
and 10^{-7} M was not change compared to that of control cells (data not shown).

DISCUSSION

In the present study, we demonstrated that raloxifene therapy for 3 and 6 months decreases LPS-stimulated production of IL-1β, IL-6, IL-12p40, IL-12p70 and TNF-α but not PHA-stimulated production of IL-4 and IFN-γ in whole blood cultures, suggesting that modulation of bone-resorbing cytokines such as IL-1β, IL-6 and TNF-α could play a role in the mechanisms of the osteoprotective effect of raloxifene. We used the whole blood culture method to determine LPS- or PHA-stimulated cytokine production in *ex vivo* and *in vitro* studies. Since isolation of PBMC by hyperosmolar solutions such as Ficoll-Hypaque and their culture *ex vivo* could modify the function of PBMC compared with that *in vivo* (19), the whole blood culture method seems to be the best method for mimicking *in vivo*

conditions of immune reactions (12).

Raloxifene therapy has been reported to have effects on the production or circulating levels of bone-resorbing cytokines such as IL-1β, IL-6 and TNF-α, but results of studies on changes in these cytokines were inconsistent. Gianni *et al*. reported that the mRNA expression of IL-6 and TNF-α was reduced by raloxifene therapy for 6 months (15). On the other hand, raloxifene treatment did not reduce circulating levels of IL-6 and TNF-α (16, 17), although Walsh *et al*. reported that raloxifene therapy significantly decreased serum TNF-α level but not IL-6 level (14). Furthermore, Rogers *et al*. demonstrated that raloxifene therapy did not modulate 500 ng/ml LPS-stimulated production of IL-1β by using a whole bloody assay (18). However, we have shown that raloxifene therapy had an inhibitory effect on LPS-stimulated production of IL-1β, IL-6 and TNF-α, which is consistent with our previous results using HRT (11). In this study, whole blood cells were stimulated by 1 μg/ml LPS, which is a higher concentration than that used by Rogers

et al. Since LPS has strong stimulatory effects that could outweigh any potential inhibitory effect of raloxifene, the reason for the difference in the production of IL-1β is unclear.

In the *in vitro* study, raloxifene at concentrations of 10^{-9}-10^{-7} M significantly inhibited LPS-stimulated production of IL-1β and IL-6 and raloxifene at concentrations of more than 10^{-8} M significantly inhibited LPS-stimulated production of TNF-α. Although we did not observe a dose-dependent response of raloxifene on IL-1β, IL-6 and TNF-α production, a raloxifene dose of 10^{-9} M was required to show a significant response. It is estimated that a raloxifene dose of 10^{-9} M is equivalent to the circulating concentration in raloxifene-treated women because the maximum plasma concentration after multiple dosing of raloxifene at 60 mg/d is 1.36 ng/ml, which is equivalent to 2.6×10^{-9} M (20). Taranta *et al*. demonstrated that raloxifene inhibited the mRNA expression of IL-1β and IL-6 at a low concentration of 10^{-10} M in *in vitro* murine osteoblasts (21). However, in human osteoblastic cells, treatment with raloxifene at doses (10^{-8}, 10^{-7}, 10^{-6} M), but not at doses (10^{-12}-10^{-9} M), resulted in a reduction in the spontaneous production of IL-1β and IL-6 (22). Furthermore, Rogers *et al*. demonstrated that raloxifene does not modulate spontaneous production of IL-1β *in vitro* by using a whole bloody assay (18). In our study, whole blood cells were stimulated by the addition of 100 ng/ml LPS after preincubation with raloxifene for 12 h because simultaneous administration of raloxifene and LPS might negate the inhibitory effect of raloxifene due to the strong stimulatory effect of LPS. The discrepancy in results of *in vitro* studies may be explained by differences in the cells cultured and the method with or without stimulation by LPS.

To the best of our knowledge, this is the first study showing that raloxifene inhibited the production of IL-12 *ex vivo* and *in vitro*. IL-12 is known to be an inflammatory cytokine produced primarily by phagocytic cells and antigen-presenting cells. IL-12 plays a role in the early inflammatory response to infection and in the generation of T helper type 1 (Th1) cells while inhibiting the generation of Th2 cells (23). IL-12 is composed of a heavy chain (p40) and a light chain (p35), forming a disulfite-linked heterodimer (p70) (24). IL-12p40 is produced primarily by activated monocytes/macrophages, while IL-12p35 is produced constantly. In this study, raloxifene therapy for 6 months significantly reduced LPS-stimulated production of IL-12p40 and IL-12p70, although a concentration (10^{-9} M) of raloxifene inhibited LPS-stimulated production of IL-12p40 but not that of IL-12p70. On the other hand, it has been shown that 17β-estradiol at a pregnancy concentration significantly inhibited the production of IL-12p70 in whole blood stimulated with a mixture of 1 µg/ml LPS and 5 µg/ml PHA (8). Elenkov *et al*. also demonstrated that whole blood IL-12 production in pregnant women was decreased 2-fold compared with that in women during the postpartum period (25), suggesting that raloxifene as well as 17β-estradiol has an inhibitory effect on the production of IL-12.

Th1 cells have been found to produce IFN-γ and IL-2, whereas Th2 cells produce different cytokines such as IL-4 and IL-5. It has been reported that raloxifene therapy caused a decrease in serum IL-4 levels (26). In our *in vitro* study, raloxifene inhibited 1 µg/ml PHA-stimulated production of IL-4 and IFN-γ in a dose-dependent manner. However, in our *ex vivo* study, raloxifene therapy did not modulate 10 µg/ml PHA-stimulated production of IL-4 and IFN-γ by whole blood cells. In previously published articles, we have shown that HRT had inhibitory effects on the production of IL-4 and IFN-γ in 10 µg/ml PHA-stimulated cultures (9, 10). Since 10 µg/ml PHA might negate the inhibitory effect of raloxifene due to the strong stimulatory effect of PHA, further study is needed to clarify the effect of raloxifene therapy on PHA-stimulated production of IL-4 and IFN-γ.

In conclusion, we have shown that raloxifene therapy and a raloxifene compound have inhibitory effects on the production of bone-resorbing or proinflammatory cytokines including IL-1β, IL-6, IL-12 and TNF-α, suggesting that raloxifene may play a role in bone resorption and formation due to suppression of the production of these cytokines.

ACKNOWLEDGMENTS

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