Article

Effects of Transdermal Estrogen Therapy on Expressions of Estrogen Receptors and T-lymphocyte Apoptosis in Surgically Menopausal Women

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Studies have demonstrated estrogen replacement therapy can improve the life quality of surgically menopausal women. However, the mechanisms in this process remain poorly defined. Here we show the effect of transdermal estrogen therapy on expressions of estrogen receptors and T-lymphocyte apoptosis in surgically postmenopausal women. Fifteen surgically menopausal women, 15 naturally menopausal women and 15 young women were chosen in our studies. Peripheral vein blood was collected and serum E_2 and FSH levels were assessed using ACCESS. T-lymphocyte apoptosis and the expressions of Fas, FasL and ER subtypes α and β were determined. The serum E_2 levels of surgically menopausal woman were significantly higher, and the "Improved Kupperman Index" and the scores of "Menopause Specific Quality of Life Questionnaire" in surgically menopausal women were significantly low after ERT. The rates of T-lymphocyte apoptosis and FasL expression in surgically menopausal women were decreased after ERT, but the difference was not significant. The expressions of ERα and ERβ in two menopausal groups were significantly lower than those of the young group. They were both significantly up-regulated after 3 months of ERT. Transdermal ERT could significantly upregulate the serum E_2 level, could improve menopausal symptoms and life quality of surgically menopausal women and upregulate ERα and ERβ expressions on T lymphocytes, especially ERβ. Thus, the low dose of transdermal ERT may have a protective effect on menopausal women's immune function and aging. *Cellular & Molecular Immunology*. 2009;6(4):277-283.

Key Words: estrogen replacement therapy, surgical menopause, estrogen receptor, T-lymphocyte apoptosis

Introduction

The prolongation of life expectancy during this century has resulted in the post-menopausal years constituting more than one-third of the lifespan of most women. With the decline of sex hormone levels after menopause, menopausal women may suffer from many disorders resulting from the deficiency of estrogen (such as vasomotor symptoms), genitourinary tract atrophy, osteoporosis, and so on. As an immunomodulator, estrogen has been recognized for many years, the deficiency of estrogen after menopause may contribute to the decline of immune function, resulting in increasing incidence rate of infectious diseases, autoimmune diseases and tumors in post-menopausal women. However, few studies focused on the difference in immune function between surgically menopausal women (who had undergone total hysterectomy and bilateral salpingo-oophorectomy because of benign uterus diseases) and naturally menopausal women (healthy women in their natural post-menopausal period). The hormonal profiles of these two types of menopausal women are different.

Hormone replacement therapy (HRT) is widely used to prevent or alleviate menopause-related symptoms and disorders. Clinical literature suggests that the use of estrogen after menopause may have substantial beneficial effects in relieving vasomotor symptoms (1), reducing skin and reproductive tract atrophy (2), delaying loss of cognitive function (3), and preventing bone losses (4). A lot of research also focused on the effect of estrogen on post-menopausal immune function (5-7), but some of the results are contradictory. Transdermal estrogen therapy, due to its less adverse effects by avoiding the hepatic first-pass effect, is a good choice for HRT. The effect of transdermal estrogen on immune function of surgically post-menopausal women is to

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Table 1. General characteristics of the three groups

Group (n = 15)	Naturally menopausal	Surgically menopausal	Pre-menopausal
Age (year)	53.7 ± 1.6	52.8 ± 2.5	27.9 ± 2.19
Months of menopause	32.1 ± 2.5	28.8 ± 3.8	—
Body mass index (kg/m^2)	25.8 ± 2.8	26.2 ± 3.3	20.8 ± 2.0
Waist-to-hip ratio	0.84 ± 0.11	0.85 ± 0.09	0.71 ± 0.04

be investigated.

T lymphocyte is one of the most important adaptive immunocytes. Apoptosis of T lymphocytes is an important way to clear antigen-activated and self-immunoactive T lymphocytes to prevent immunological injuries and maintain organism's normal immune function. After menopause, with the deficiency of estrogen and physical aging, some changes may occur to the apoptosis of T lymphocytes.

Several reports have addressed the expressions of two estrogen receptors (ERs), ERα and ERβ, on human T lymphocyte (8, 9). However, little is known regarding the relation of ERT, ER expression on T lymphocytes and apoptosis of T lymphocytes.

In this study, we studied the expressions of estrogen receptors and T-lymphocyte apoptosis in surgically menopausal women before or after transdermal estrogen therapy. The studies demonstrate that the expressions of ERα and ERβ on T lymphocytes are increased and T lymphocyte apoptosis and FasL expression are decreased after transdermal ERT. It indicates low dose of transdermal ERT may have a protective effect on menopausal women's immune function.

Materials and methods

Patients

This study has been approved by Ethical Committee of OB/GYN Hospital, all patients were given an informed consent before initiation of the study. Fifteen surgically menopausal women aged 48 to 57 years were selected, who have undergone total hysterectomy and bilateral salpingo-oophorectomy in our hospital for uterine myoma within 1 to 5 years. They were qualified for the study on the basis of indication of ERT (e.g. severe menopausal symptoms, urogenital atrophy, etc) and no contraindication of ERT. Another fifteen women who were at their natural menopausal period for 1 to 5 years were selected as a control group. The surgically menopausal group and naturally menopausal group were matched for age, years since menopause, body mass index, and waist-to-hip ratio. The second control group consisted of 15 women who had normal menstrual circles, aged 25 to 35 and blood collected at their follicle phases. All menopausal women had no history of HRT during the three previous months. All women selected in the study had no history of auto-immune diseases, and were not given any medical therapy known to affect the immune system for at least 1 year before and during the onset of the study. The general characteristics of the three groups are shown in Table 1.

The surgically menopausal group was given transdermal estradiol gel for smearing (1.25 g/day, consisting of 0.75 mg 17β-estradiol, Wuhan Jianmin Pharmacy Corporation Limited, China) for 3 months. All surgically menopausal women took general gynecologic, mammary examination, pelvic B ultrasound investigation and serum sex hormone levels assay before and after ERT. Improved Kupperman Index and Menpause-Specific Quality of Life Questionnaire were used to evaluate the menopausal complaints and life qualities before and after ERT. Thirteen women completed 3 months of ERT.

The two control groups were given no intervention. For assay of hormone levels and isolation of T lymphocytes, 15 ml peripheral blood samples were taken from each woman. About 8-9 ml bloods was immediately heparinized (25 U/ml) for T lymphocytes isolation, and the left blood was used for serum hormone levels and other indices assay.

Isolation and culture of T lymphocytes

RosetteSep Human CD3$^+$ T Cell Enrichment Cocktail (Stemcell, Canada) was added to heparinized whole blood at 50 μl/ml, incubate for 20 min at room temperature, and CD3$^+$ T cells were isolated by Ficoll-Paque Plus density medium (Stemcell, Canada) following manufacturer's instructions.

About 4-5 × 10^6 CD3$^+$ T lymphocytes were isolated from each individual and adjusted to 1 × 10^6 cells/ml with

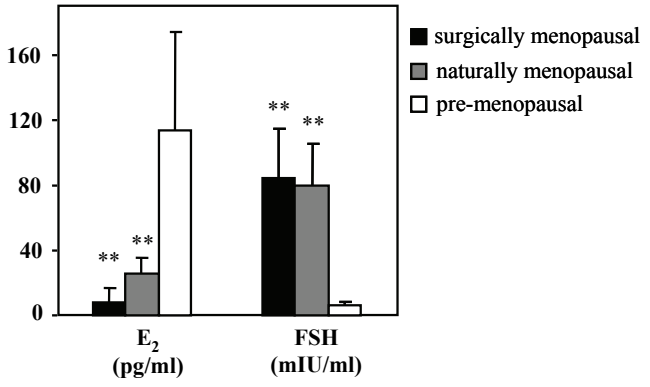

Figure 1. The levels of E2 and FSH in serum. The peripheral vein blood of the subjects from three groups (surgically menopausal group, naturally menopausal group and pre-menopausal group) was collected, and the serum hormone levels were detected. Data were shown as mean ± SD, n = 15. $^{**}p < 0.01$.

Table 2. Improved Kupperman Index and Menopause Specific Quality of Life Questionnaire of surgically menopausal group before and after ERT.

	Before ERT	After ERT
Improved Kupperman Index	19.46 ± 6.70	14.46 ± 4.46*
Menopause Specific Quality of Life Questionnaire	37.31 ± 8.92	23.77 ± 9.69*

*$p < 0.01$, compared with that of ERT early

RPMI1640 medium (Gibco, USA) containing 10% FCS (Gibco, USA). T cells were inoculated to 96-well plates and 24-well plates coated with 5 μg/ml anti-CD3 monoclonal antibody (BD Biosciences, USA) respectively, then anti-CD28 monoclonal antibodies (BD Biosciences, USA) were added to each well at a concentration of 1 μg/ml. Following 72 h culture, T lymphocytes were collected. Cells in 96-well plates were used for assay of T cell apoptosis; cells in 24-well plates were used for protein extraction and Western blot analysis.

Evaluation of serum hormone levels

Serum levels of E_2 and FSH were routinely quantitated by specific auto-immunochemiluminescent assays (Beckman Coulter, USA) according to a detailed description provided by the manufacturer.

Assays of T-cell apoptosis

After 72 h of culture, cells were harvested and washed twice with cold PBS. Detection of apoptosis was performed using Annexin V-FITC/PI apoptosis detection kit (Bender, USA) following manufacturer's instructions. Apoptotic cells were recognized as Annexin V+/PI-.

Measurement of ER，ERβ，Fas and FasL protein expressions

The protein expressions were determined by Westernblot. Aliquots of total protein (20 μg/lane) were electrophoresed on 12% PAGE gels and transferred to PVDF membranes (Millpore, USA). The membranes were incubated overnight at 4°C with mouse anti-human ERα monoclonal antibody (Stressgen, USA), mouse anti-human ERβ monoclonal antibody (abcam, USA), rabbit anti-human Fas and FasL polyclonal antibodies (Santa Cruz, USA), mouse anti-human β-actin monoclonal antibody (abcam, USA), respectively. After washing with rinse buffer, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin antibodies. Immuno-reactive proteins were visualized using an enhanced chem.-iluminescence detection system (Beyotime, Jiangsu, China). The intensity of protein bands were quantitated using Quantity One Image analysis system (Bio-Rad, USA).

Statistical analysis

Results were expressed as means ± SD. Intergroup comparisons were made using one-way analysis of variance (ANOVA), and before-and-after-treatment comparisons were made using paired *t* test. P values less than 0.05 were considered statistically significant.

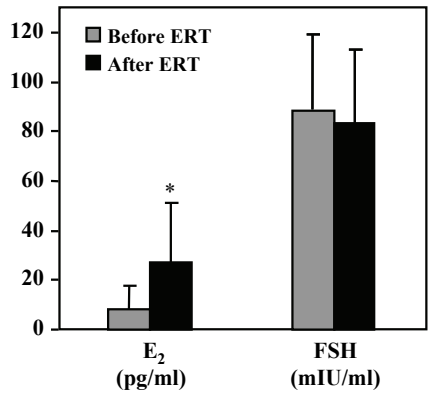

Figure 2. The levels of E2 and FSH in serum before and after ERT. The peripheral vein blood of the subjects from surgically menopausal group before or after ERT) was collected, and the serum hormone levels were detected. Data were shown as mean ± SD, n = 13. *$p < 0.05$.

Results

Comparison of serum hormonal levels in the patients

The serum E_2 level of surgically and naturally menopausal groups were significantly lower than that of pre-menopausal group ($p < 0.01$), and the FSH level much higher than that of pre-menopausal group ($p < 0.01$). It was a trend that E2 level of naturally menopausal group was higher than surgically menopausal group, but the difference was not significant. The FSH level between surgically group and naturally menopausal group were not significantly different (Figure 1). The E_2 level of surgically menopausal group was significantly increased after 3 months of ERT ($p < 0.05$). There was a decreasing trend of FSH level after ERT, but the difference was not significant (Figure 2).

Effect of ERT in surgically menopausal patients

The clinical statuses of surgically menopausal group before and after ERT were determined with Improved Kupperman Index and Menopause Specific Quality of Life Questionnaire. ERT resulted in a significant improvement of both the two indices ($p < 0.01$), as shown in Table 2.

T cells apoptotic rates in the patients

The T lymphocyte apoptotic rates of surgically and naturally menopausal groups were both much higher than that of pre-menopausal group ($p < 0.05$). There was no significant difference between surgically menopausal group and naturally menopausal group (Figure 3). There was a decreasing trend of T cell apoptotic rate of surgically menopausal group after ERT compared with before, but the difference was not significant (Figure 4).

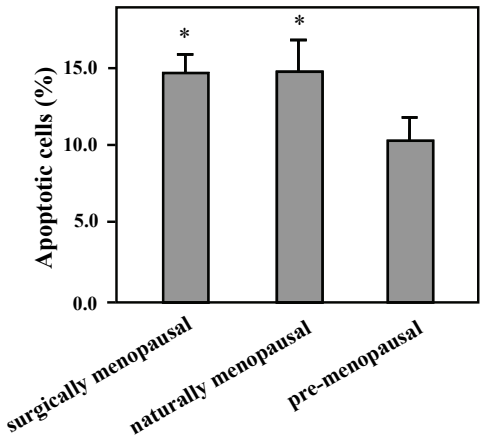

Figure 3. Apoptotic rates of T cells. T lymphocytes of patients from surgically menopausal group, naturally menopausal group and pre-menopausal group, were isolated and cultured, and the Annexin V+/PI- cells were detected by flow cytometry. Data were shown as mean ± SD, n = 15. *$p < 0.05$.

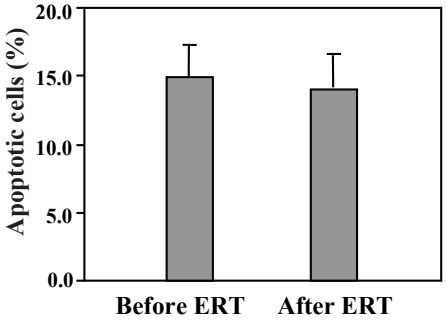

Figure 4. Apoptotic rates of T cells before and after ERT. T lymphocytes of patients from surgically menopausal group were isolated and cultured before and after ERT, and the Annexin V+/PI- cells were detected by flow cytometry. Data were shown as mean ± SD, n = 13.

Expressions of Fas and FasL proteins on T lymphocytes in the patients

The Fas and FasL protein expressions in T lymphocytes of surgically and naturally menopausal groups were both significantly higher than that of pre-menopausal group ($p < 0.05$). There was no significant difference between surgically menopausal group and naturally menopausal group (Figure 5). After 3 months of ERT, the Fas protein expression on T lymphocyte in surgically menopausal women was not changed. The FasL protein expression was decreased after ERT, but the difference was not significant (Figure 6).

Expression of ERα and ERβ proteins on T lymphocytes in the patients

The ERα protein levels in T lymphocytes of surgically (before ERT) and naturally menopausal group were both significantly lower than that of young group ($p < 0.05$); it was the same with ERβ protein levels ($p < 0.01$, $p < 0.05$, respectively). There was no difference of ERα and ERβ protein levels between surgically group and naturally menopausal group (Figure 7). After ERT, the ERα and ERβ protein levels in T lymphocytes of surgically menopausal group were both significantly higher than early ERT ($p < 0.05$, $p < 0.01$, respectively) (Figure 8).

Discussion

Our study confirmed the salutary effect of transdermal estradiol gel in improving menopause-related symptoms, which is consistent with the results of Archer and Akhila V, Simon JA, etc (1, 10, 11). In transdermal ERT, estrogen is absorbed by skin and released slowly into blood, avoiding the elimination of liver and making transdermal HRT suitable for menopausal women who can't take oral HRT with chronic hepatic disease or coagulation disorders and gastrointestinal tract diseases. The International Menopause Society (IMS) also recommended the use of non-oral estrogens for those at increased risk of venous thromboembolism (12). In this study, the transdermal estradiol gel was acceptable to most patients,

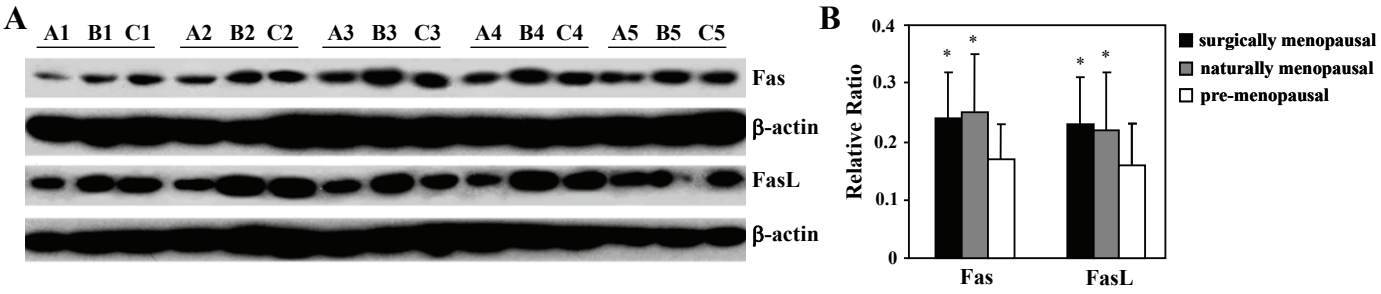

Figure 5. Expressions of Fas and FasL on T lymphocytes in the patients. T lymphocytes of patients from three groups (surgically menopausal group, naturally menopausal group and pre-menopausal group) were isolated. (A) Expressions of Fas and FasL on T lymphocytes were determined by Western blot. A1-A5, B1-B5, C1-C5 respectively stand for 5 subjects of pre-menopausal group, naturally menopausal group and surgically menopausal group. (B) The relative ratios were calculated and data were shown as mean ± SD. *$p < 0.05$.

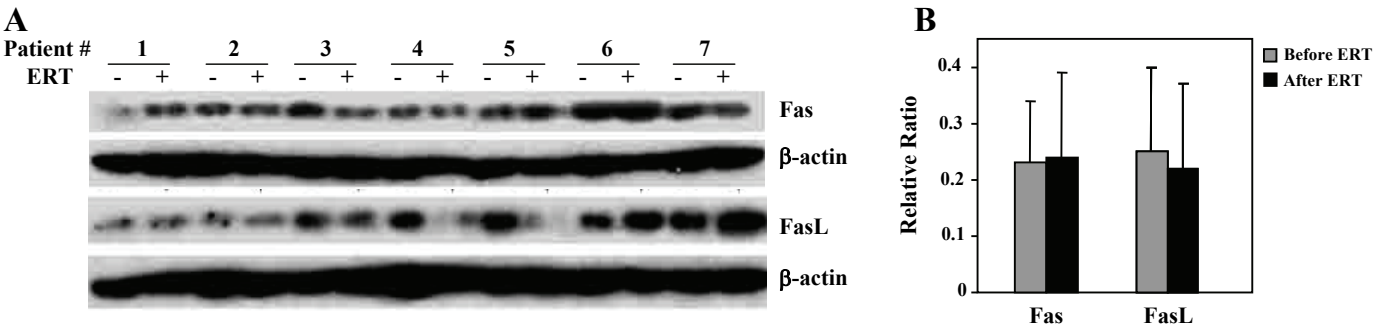

Figure 6. Expressions of Fas and FasL on T lymphocytes before and after ERT. T lymphocytes of patients from surgically menopausal group were isolated before and after ERT. (A) Expressions of Fas and FasL on T lymphocytes were determined by Western blot. Lans 1-7 respectively stand for 7 subjects. (B) The relative ratios were calculated and data were shown as mean ± SD.

causing very few adverse effects.

As one of the most important adaptive immunocytes in the organism, T lymphocyte's normal function is pivotal for the organism's normal immune responsiveness. Apoptosis is one important property of T lymphocytes, through which antigen-activated and defective T lymphocytes are depleted to maintain normal immune responsiveness. The abnormal death of aging T lymphocytes may result in the defective anti-infection and anti-tumor ability of the organism. The decline of E_2 level and physical aging after menopause may affect the apoptotic ability of T lymphocytes. Many studies had reported the increased apoptotic susceptibility of aging T cells (13-15). In our study, the T lymphocyte apoptotic rate of post-menopausal women was significantly higher than that of pre-menopausal women, in consistent with the previous results. There was no significant difference in T cell apoptosis between the two post-menopausal groups. Along with the increased apoptosis of T lymphocytes, post-menopausal symptoms and decreased life quality occurred on post-menopausal women, indicating a possible relationship between excessive T cell death and impaired life quality after menopause.

Regarding the effect of estrogen on human T lymphocyte apoptosis, there are few reports and some results are contradictory. Hirano S reported that physically high concentrations of 17β-estradiol inhibited the apoptosis of human peripheral T lymphocyte (16). Takao T also reported the protective effect of 17β-estradiol on human T lymphocyte (8). In another experiment using animals, supraphysiological level of estrogen increased lymphoid cell death in ovariectomized mice (17). In Porter V.R's report, the T lymphocyte apoptotic rate of post-menopausal women using HRT was higher than that of post-menopausal women not using HRT, but the difference was not significant. In this study, the T lymphocyte apoptotic rate of surgically menopausal women after 3 months of transdermal ERT was lower than that before ERT, but the difference was not significant. We used low dose of transdermal ERT，and the level of estradiol after ERT was similar to the level of early follicle phase. The post-menopausal symptoms and life quality were significantly improved after ERT. This result suggests a beneficial effect of transdermal ERT on the immune system of surgically menopausal women, although it is to be determined whether statistically significant results can be achieved or not if prolonging the study term or enlarging the subject population. Our result also suggests that

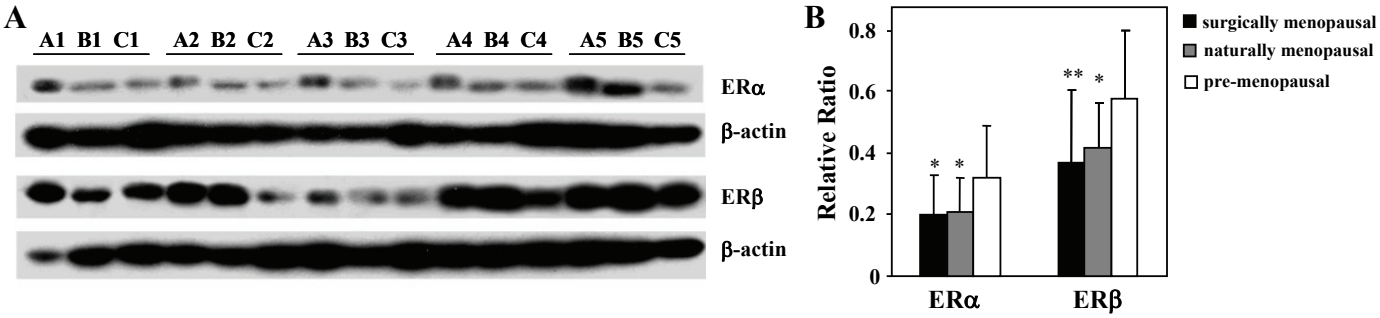

Figure 7. Expressions of ERα and ERβ on T lymphocytes. T lymphocytes of patients from surgically menopausal group, naturally menopausal group and pre-menopausal group were isolated. (A) Expressions of ERα and ERβ on T lymphocytes were determined by Western blot. A1-A5, B1-B5, C1-C5 respectively stand for 5 subjects of pre-menopausal group, naturally menopausal group and surgically menopausal group. (B) The relative ratios were calculated and data were shown as mean ± SD. *$p < 0.05$, **$p < 0.01$.

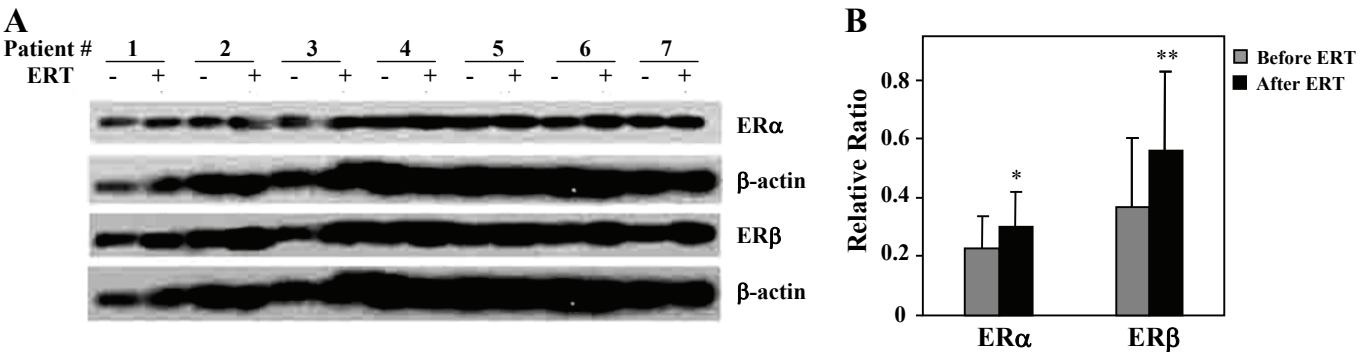

Figure 8. Expressions of ERα and ERβ on T lymphocytes before and after ERT. T lymphocytes of patients from surgically menopausal group were isolated before and after ERT. (A) Expressions of ERα and ERβ on T lymphocytes were determined by Western blot. Lans 1-7 respectively stand for 7 subjects. (B) The relative ratios were calculated and data were shown as mean ± SD. *$p < 0.05$, **$p < 0.01$.

estrogen in ERT should be used with the lowest effective dose.

Fas (the death receptor) and its ligand FasL play a pivotal role in the activation induced cell death (AICD) of T lymphocyte (18, 19). Aggarwal S reported that the increased apoptosis of T cell subsets in aging humans was related to the increased expression of Fas and FasL (20). Our study detected the increased protein expression of Fas and FasL in post-menopausal women compared to pre-menopausal women, indicating the increased apoptosis of post-menopausal women was related to the increased expression of apoptotic molecules. There exists estrogen response element (ERE) on the promoter of FasL gene (21), and estrogen can regulate the expression of FasL through binding to ER. In our study, 3 months of low-dose transdermal ERT decreased protein expression of FasL to some extent, although the difference was not significant. This indicated that low dose of ERT may be beneficial to the immune system.

Estrogen produces its effect through conjunction with ER of target cell or organ. So the change of expression of receptors may affect the biological effects of estrogen on target cell or organ. ERα and ERβ are both subtypes of ER, and they are different in construction in both N end and C end, which imply they may have different functions. ERα and ERβ have been reported to be co-expressed on human T lymphocyte (8, 9), and our study confirmed their co-expression. There are different conclusions about which ER subtype is dominantly expressed on human T lymphocytes and which subtype is mainly responsible for the effect of estrogen. We observed more ERβ protein expression than ERα on human T lymphocytes, in consistent with the results of Hirano S (16) and Shim GJ (22).

Our research has proved that the concentration of estrogen within physiological dose is positively associated with the expression of ER. In our research, the protein expressions of ERα and ERβ were both increased after 3-month long ERT, and the uplevel of ERβ was more significant. The up-regulation of ER expression may amplify the biological effect of estrogen. Through the conjunction of liganded ER (probably ERβ, concluded from the results) with ERE on FasL gene promoter, estrogen regulated the apoptosis of T lymphocytes of surgically menopausal women. However, the molecular signaling pathways of E_2 and ER are very complicated, involving many other signaling molecules. More research works are needed to do to explore the complex downstream signaling pathways.

Acknowledgments

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