Effects of oestrogen deprivation on interleukin-6 production by peripheral blood mononuclear cells of postmenopausal women

D Rachoń, J Myśliwska, K Suchecka-Rachoń[1], J Więckiewicz and **A Myśliwski**

Department of Histology and Immunology of the Medical University of Gdańsk, 80 210 Gdańsk, ul. Dębinki 1, Poland

[1]Department of Hypertension and Diabetology of the Medical University of Gdańsk, 80 211 Gdańsk, ul. Dębinki 7, Poland

(Requests for offprints should be addressed to D Rachoń; Email: drachon@amedec.amg.gda.pl)

Abstract

Various hormones can influence the expression of interleukin-6 (IL-6) and oestrogens are the most extensively studied. There is, however, controversy about the nature of the IL-6 secreted by human cells and its regulation by 17β-oestradiol. The aim of this work was to clarify whether oestrogen deprivation after menopause may contribute to an enhanced IL-6 production by peripheral blood mononuclear cells (PBMC) in postmenopausal women. Twenty-two healthy postmenopausal women, age range 45–63 years, with clinical symptoms of oestrogen deficiency were enrolled in the study. The control group consisted of 16 healthy young women, age range 22–31 years, with regular menses and who were not taking oral contraceptives. Levels of IL-6 in the sera and PBMC culture supernatants were measured by the biological B9 cell-proliferation assay and expression of the IL-6 gene in non-stimulated PBMC was detected by RT-PCR. The effect of 17β-oestradiol on spontaneous IL-6 production by the PBMC of postmenopausal women was also studied *in vitro* and *in vivo*. Seventeen out of the twenty-two postmenopausal women were given hormonal replacement therapy of 50 μg 17β-oestradiol/day transdermally and the spontaneous production of IL-6 by the PBMC was analysed after 6 and 12 months of treatment.

The postmenopausal women had significantly higher serum levels of IL-6 than the young controls. The spontaneous production of IL-6 by non-stimulated PBMC into the culture supernatants was also significantly higher in the postmenopausal women compared with the young. We also found that IL-6 gene expression was present in the non-stimulated PBMC isolated directly from the venous blood of the majority of the postmenopausal women. Women with IL-6 gene expression in the non-stimulated PBMC had significantly lower serum levels of 17β-oestradiol compared with those where the IL-6 gene was not expressed in the PBMC. Our *in vitro* experiments showed that 17β-oestradiol at concentrations of 10^{-9} M and 10^{-10} M decreased spontaneous IL-6 production by the PBMC of postmenopausal women. *In vivo* treatment with 17β-oestradiol transdermally also significantly decreased spontaneous IL-6 production by the PBMC of postmenopausal women after 12 months of the therapy.

Our results indicate that oestrogen deprivation after menopause may enhance IL-6 production by the PBMC of postmenopausal women. We suspect that the late complications of oestrogen deficiency, such as osteoporosis, coronary heart disease and Alzheimer's disease, may be mediated by an exaggerated production of IL-6 – a cytokine which seems to play a pivotal role in the pathogenesis of these age-related diseases.

Journal of Endocrinology (2002) **172**, 387–395

Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine produced by immune and non-immune cells and organs. Unlike other cytokines, IL-6 is unusual in that its major effects take place at sites distinct from its origin and are consequent upon its circulating concentrations. For this reason, it is called the endocrine cytokine (Papanicolaou & Vgontzas 2000). Inappropriate expression and production of IL-6 is thought to be involved in the pathogenesis of numerous diseases, including osteoporosis (Manolagas & Jilka 1995),

coronary heart disease (CHD) (Huber *et al.* 1999) and Alzheimer's disease (AD) (Mrak *et al.* 1995). Conversely, these disorders belong to the late complications of menopause and oestrogen replacement therapy may improve them (Lindsay *et al.* 1984, Stampfer & Colditz 1991, Tang *et al.* 1996).

There is, however, controversy about the nature of the IL-6 secreted by human cells and its regulation by oestrogens. Girasole *et al.* (1992) demonstrated that 17β-oestradiol inhibits IL-6 production in several types of osteoblastic cells *in vitro* including murine stromal

cell lines, murine osteoblasts and human osteoblasts. In addition, oestrogens have been shown to inhibit IL-1β- and tumour necrosis factor (TNFα)-α-stimulated IL-6 secretion by the human fetal osteoblast cell line (hFOB/ER9) which expresses a high level of oestrogen receptors (Kassem *et al.* 1996a). Conversely, oestrogen loss results in increased IL-6 production by *ex vivo* bone marrow cell cultures (Passeri *et al.* 1993), and increased IL-6 production follows withdrawal of 17β-oestradiol from primary cultures of calvarial cells (Passeri *et al.* 1994). *In vivo* studies also showed that women taking hormone replacement therapy (HRT) have significantly lower serum IL-6 levels compared with subjects not taking HRT (Straub *et al.* 2000).

In contrast, subsequent studies by three groups (Chaudhary *et al.* 1992, Rickard *et al.* 1992, Rifas *et al.* 1995) could not demonstrate a regulatory effect of ovarian steroids on IL-6 secretion in animal and human bone cell systems. In addition, McKane *et al.* (1994) did not find any differences in serum IL-6 levels when comparing pre- and postmenopausal women, and there was no difference in IL-6 levels in bone marrow aspirates from postmenopausal women with or without HRT (Kassem *et al.* 1996b).

In view of these contradictory findings obtained in different cell models, the present study was carried out to clarify whether oestrogen deprivation and HRT have an effect on the production of IL-6 by the peripheral blood mononuclear cells (PBMC) of postmenopausal women.

We have compared serum levels of bioactive IL-6 as well as spontaneous IL-6 production into the supernatants by non-stimulated PBMC in young and postmenopausal women. We have also studied expression of the IL-6 gene in non-stimulated, freshly isolated PBMC of both groups. The *in vitro* and *in vivo* effects of 17β-oestradiol on spontaneous IL-6 production by the PBMC of postmenopausal women were also studied.

Materials and Methods

Subjects and study design

Twenty-two healthy postmenopausal women, age range 45–63 years, from the Endocrinological Outpatient Clinic of the Medical University of Gdańsk were enrolled in the study. Postmenopausal status was defined as an absence of menstrual periods for at least the previous 6 months and the presence of the oestrogen deficiency symptoms ('hot flushes', increased sweating, nervousness, irritability, depression, palpitations, insomnia, headaches, dyspareunia and joint pains). Four women had experienced surgical menopause. The time since menopause ranged from 6 months to 13 years. The cessation of ovarian function was confirmed by the measurement of serum follicle-stimulating hormone (FSH) levels which were all above 40 IU/l. The control group consisted of 16 healthy young

women, age range 22–31 years, with regular menses and who were not taking oral contraceptives.

All participants underwent a routine physical examination and routine biochemical screening, including complete blood cell count and chemical group typing. Women from both groups were clinically healthy and were not taking any medication known to affect immunological status, and in the last 2 weeks had had no apparent acute or chronic inflammatory diseases that could result in the elevation of IL-6 levels. Moreover, we selected only those postmenopausal women who were free from osteoporosis (on the basis of bone mass density measurements by X-ray absorptiometry), CHD (on the basis of electrocardiography, biochemical screening and clinical status) and signs of dementia.

All subjects were informed about the purpose of the study and gave written consent. After a detailed talk with the endocrinologist (K Suchecka-Rachoń) about the benefits and risks of HRT, 17 out of 22 postmenopausal women were treated with 50 μg 17β-oestradiol/day transdermally (Estraderm 50MX; Novartis PharmaAG, Basel, Switzerland). Women with an intact uterus (13 subjects) were also receiving 10 mg medroxyprogesterone acetate (Provera; Pharmacia and Upjohn, Ascoli Piceno, Italy) for 10 days during each cycle. Venous blood was taken at baseline and after 6 and 12 months of HRT when patients were not taking the medroxyprogesterone acetate treatment. Blood from the young subjects was drawn between the 5th and 10th day of their menstrual cycle. These studies were approved by the Ethic Committee of the Medical University of Gdańsk.

Sera

Venous blood was drawn between 0900 and 1000 h and serum was immediately stored at − 80 ˚C, in suitably sized aliquots, until thawed for analysis.

Isolation of PBMC

For cell culture and RNA isolation, blood was drawn on EDTA. Then the PBMC were isolated by centrifugation (2500 r.p.m. for 15 min at 20 ˚C) using Ficoll–Paque PLUS (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The interphase containing the PBMC was collected and washed twice (1500 r.p.m. for 7 min at 20 ˚C) in phosphate-buffered saline (Gibco BRL, Life Technologies, Berlin, Germany).

PBMC cell cultures

Non-stimulated PBMC were incubated in RPMI 1640 (Gibco BRL, Life Technologies) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Gibco BRL, Life Technologies) at about 1×10^6 cells/ml on a 24-well plate (Nunc A/S, Roskilde, Denmark). After 24 h of incubation

in a humidified atmosphere containing 5% CO_2 at 37 °C, the supernatants were collected and stored at -80 °C in aliquots until thawed for analysis.

PMBC of postmenopausal women were also incubated with different concentrations of 17β-oestradiol (Sigma, Steinheim, Germany). 17β-Oestradiol was dissolved in absolute ethanol and prepared as a stock solution of 10^{-4} M. The cells were plated in RPMI 1640 supplemented with 5% heat-inactivated FCS at about 1×10^6 cells/ml on a 24-well plate. The hormone stock solution was diluted serially in RPMI 1640 before addition to the cells, and added to culture medium to yield 10^{-8} to 10^{-11} M. Ethanol at a 1:1000 dilution was used as the vehicle control. After 24 h of incubation in a humidified atmosphere containing 5% CO_2 at 37 °C, the supernatants were collected and stored at -80 °C in aliquots until thawed for analysis.

Biochemical assays

Serum FSH and 17β-oestradiol measurements were carried out in a diagnostic laboratory using the commercially available Abbott AxSYM assay (Abbott Park, IL, USA) based on microparticle enzyme immunoassay technology.

Isolation of RNA

After isolating the PBMC from the whole blood, total RNA was extracted with Trizol Reagent (Gibco BRL, Life Technologies Inc., Frederick, MD, USA) according to the manufacturer's instructions. The quantity and quality of RNA were tested spectrophotometrically using A_{260}/A_{280} on the UV/VIS Spectrometer MBA 2000 (Perkin Elmer, Norwalk, CT, USA). Additionally, RNA samples were stained with ethidium bromide and ribosomal RNAs (28s and 18s) were visualized on a 1% agarose gel (Promega, Madison, WI, USA) by the UV transillumination technique (UVP, Upland, CA, USA). Nondegraded RNA was selected for further processing.

RT-PCR

cDNA was synthesized from 1 μg total RNA in a 20 μl reaction mixture containing 5 μl 5 × reverse transcription buffer which contained 15 mM $MgCl_2$, 1·25 μl 10 mM dNTP mixture, 0·5 μl ribonuclease inhibitor RNasin, 200U M-MLV reverse transcriptase and 1 μl random primers. All reagents were purchased from Promega. This solution was incubated in a TGradient Thermocycler Modul 96 (Biometra GmbH, Göttingen, Germany) at 42 °C for 60 min and then heated at 99 °C for 5 min.

Aliquots of 3 μl of the total cDNA were amplified in a 33 cycle PCR in a 50 μl reaction mixture containing 5 μl 10 × PCR buffer which contained 15 mM $MgCl_2$, 38·2 μl sterile H_2O, 1 μl dNPT mixture (all from Promega), 1 μl IL-6 5′-primer, 1 μl IL-6 3′-primer

(Clontech Laboratories, Palo Alto, CA, USA) and 1·5 U (0·75 μl) DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland). The primers used for PCR were as follows: 5′-primer ATG AAC TCC TTC TCC ACA AGC GC; 3′-primer G AAG AGC CCT CAG GCT GGA CTG. IL-6 cDNA which was provided with the primers by the manufacturer was used as a positive control, and sterile H_2O as a negative control in the test. In all the RT-PCR experiments, controls for RNA integrity and the proper course of the RT reaction consisted of parallel cDNA samples run with β-actin primers. PCR amplification was carried out in a TGradient Thermocycler Modul 96 begining at 95 °C for 5 min, then 30 s at 95 °C, 45 s at 55 °C, 1 min at 72 °C for 33 cycles and 7 min at 72 °C. The products of the reaction were cooled to 4 °C, stained with ethidium bromide, sized in a 2% agarose gel (Promega) and visualized on a transilluminator (UVP, Germany). Gels were photographed using a polaroid system and processed by the program Grab-It (UVP, Upland, USA).

IL-6 bioassay

IL-6 levels in the sera and in the PBMC culture supernatants were determined using the B9 hybrydoma proliferation assay (Aarden *et al.* 1987). In this assay, 10×10^3 B9 cells (a gift from L Aarden, Netherland Red Cross, Amsterdam, The Netherlands) were cultured on a 96-well plate with 50 times diluted samples of sera or supernatants in a final volume of 100 μl RPMI 1640, supplemented with 5×10^{-5} M 2-mercaptoethanol (Sigma), 10% FCS, 100U/ml penicillin and 100 μg/ml streptomycin (Sigma). All samples were put onto a 96-well plate in triplicate. After 48 h of incubation in a humidified atmosphere containing 5% CO_2 at 37 °C, 20 μl (3-[4,5-Dimethylthiazol-2-yl]-2,5)-diphenyltetrazolium bromide (MTT) (Sigma) was added to each well of the plate. The plate was incubated for the next 4 h in the above conditions and after this time 100 μl isopropanol (Sigma) was added. Optical density was read at 570 nm on an automated plate reader (Bio-Tek FL600; Bio-Tek Instruments Inc., Winooski, VT, USA). A standard curve relating cell proliferation to doses of recombinant human IL-6 (Sigma) was used to quantify IL-6 activity in the samples.

To confirm the specificity of the test, the monoclonal anti-IL-6 antibody (Genzyme, Rüsselsheim, Germany) was added (1:10, 1:20, 1:50) to the samples. The anti-IL-6 monoclonal antibody completely inhibited the ability of the B9 cells to proliferate. This assay has a detection limit of 1 pg/ml. The intra-assay coefficient of variation ranged between 9·5 and 10·9% and the interassay coefficient of variation between 17·7 and 25%.

Statistical analysis

All data are presented as arithmetic means ± s.D. The differences between age groups were evaluated using

Table 1 Characteristics of the subjects studied

	Postmenopausal women	Young women
No. of subjects	22	16
Age (years)	52·77 ± 4·59*	26 ± 3·09
Years since menopause	4·48 ± 4·26	—
Serum FSH levels (IU/l)	68·23 ± 21·17*	17·63 ± 8·25
Serum 17β-estradiol levels (pmol/l)	85·66 ± 76·33*	259·77 ± 132·89

*$P<0·05$.

Student's unpaired t-test. Student's paired t-test was used to compare the *in vitro* as well as the *in vivo* 17β–oestradiol-induced effects on the spontaneous IL-6 production by the PBMC of postmenopausal women. P values $<0·05$ were considered statistically significant. All analyses were performed using a computer program STATISTICA Edition '99 for Windows.

Results

Clinical characteristics of the subjects studied

All participants underwent a routine physical examination and biochemical screening which did not reveal any abnormalities. The characteristics of the two groups studied are shown in Table 1.

As shown in Table 1, the postmenopausal women had significantly lower levels of serum 17β–oestradiol (85·66 ± 76·33 pmol/l vs 259·77 ± 132·89 pmol/l, $P<0·05$) and significantly higher serum levels of FSH (68·23 ± 21·17 IU/l vs 17·63 ± 8·25 IU/l, $P<0·05$) compared with the young control group. These differences confirmed the state of oestrogen deficiency in the postmenopausal women.

Serum levels of IL-6 in postmenopausal and young women

In order to find the differences between the levels of IL-6 in the sera of the postmenopausal and young women, the bioactive IL-6 was detected using the B9 cell-proliferation assay. It appeared that the mean serum levels of IL-6 in the postmenopausal women was significantly higher compared with the young women (4·44 ± 2·10 pg/ml vs 1·76 ± 1·38 pg/ml, $P<0·05$) (Fig. 1).

Spontaneous IL-6 production by the PBMC of postmenopausal and young women into the culture media

In the next experiment, the levels of bioactive IL-6 were compared in the supernatants from the cultures of the non–stimulated PBMC of the postmenopausal and young women. Non–stimulated PBMC from both groups were incubated in a medium supplemented with FCS in a humidified atmosphere with 5% CO_2 for 24 h. After the incubation period, the levels of IL-6 were measured in the supernatants. The results of this experiment revealed that the cultured, non–stimulated PBMC from the post–menopausal women released significantly higher amounts of bioactive IL-6 into the culture media than those of the young (13·25 ± 9·01 pg/ml vs 6·3 ± 4·23 pg/ml, $P<0·05$) (Fig. 2).

IL-6 gene expression in the non-stimulated PBMC of postmenopausal and young women

Increased release of bioactive IL-6 by the non–stimulated PBMC of the postmenopausal women into the culture supernatants suggested the possibility of an endogenous activation of these cells *in vivo*. In order to clarify this suggestion, expression of the IL-6 gene in the PBMC, isolated directly from venous blood, was studied using the RT-PCR method. The results revealed that a spontaneous expression of the IL-6 gene was present in the PBMC of 15 out of the 22 postmenopausal women (Fig. 3), and was absent in the PBMC of all the young women (data not shown).

These results confirmed an endogenous activation of the IL-6 gene in the PBMC among the majority of the postmenopausal women studied.
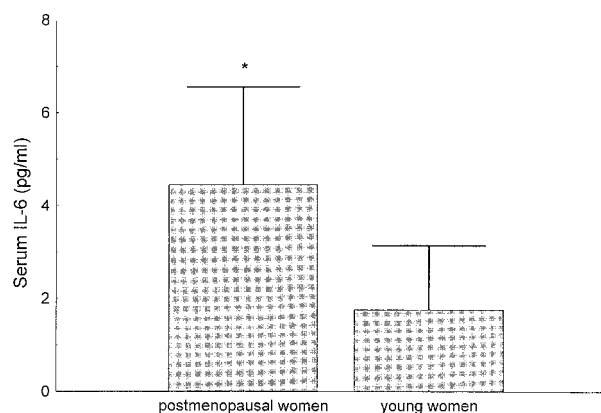

Figure 1 Serum levels of IL-6 in postmenopausal and young women. The levels of the bioactive IL-6 in the sera were measured using a B9 cell-proliferation assay. The postmenopausal women ($n=22$) had significantly higher levels of bioactive IL-6 than the young ones ($n=16$) (4·44 ± 2·10 pg/ml vs 1·76 ± 1·38 pg/ml) (*$P<0·05$).
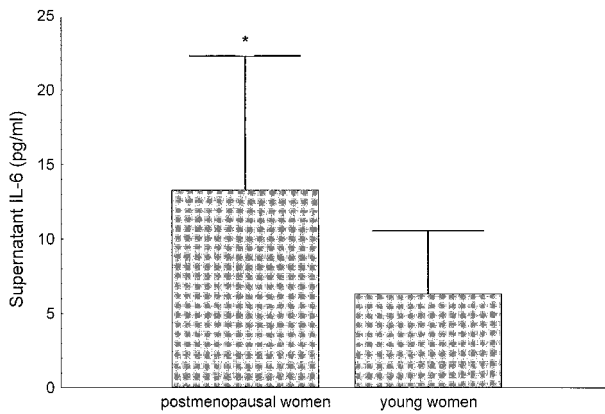
Figure 2 Levels of IL-6 in supernatants from PBMC cultures of postmenopausal and young women. Non-stimulated PBMC from the postmenopausal and young women were incubated in RPMI 1640, supplemented with 5% FCS, in a humidified atmosphere with 5% CO_2. After 24 h of incubation, levels of bioactive IL-6 were measured in the supernatants. Non-stimulated PBMC from the postmenopausal women ($n=22$) released into the supernatants significantly higher amounts of IL-6 compared with the PBMC of the young women ($n=16$) ($13·25 \pm 9·01$ pg/ml vs $6·3 \pm 4·23$ pg/ml) (*$P<0·05$).

Figure 4 Serum 17β-oestradiol levels and expression of the IL-6 gene in the non-stimulated PBMC of postmenopausal women. Postmenopausal women with IL-6 gene expression ($n=15$) in the non-stimulated PBMC, isolated directly from venous blood, had significantly lower serum 17β-oestradiol levels compared with those who did not express the IL-6 gene in the PBMC ($n=7$) ($59·05 \pm 49·62$ pmol/l vs $156 \pm 86·65$ pmol/l) (*$P<0·05$).

Serum 17β-oestradiol levels and constitutive IL-6 gene expression in the PBMC of postmenopausal women

When the relationship between the levels of 17β–oestradiol and IL-6 gene expression in the non–stimulated PBMC was analysed, it appeared that a constitutive IL-6 gene expression was found in those postmenopausal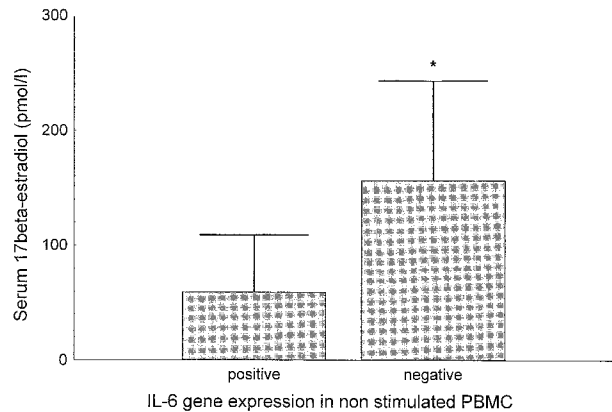 women who had significantly lower mean serum levels of 17β–oestradiol compared with those who did not show a constitutive expression of the IL-6 gene in their PBMC ($59·05 \pm 49·62$ pmol/l vs $156 \pm 86·65$ pmol/l, $P<0·05$) (Fig. 4).

In vitro effects of 17β-oestradiol on spontaneous IL-6 production by the PBMC of postmenopausal women

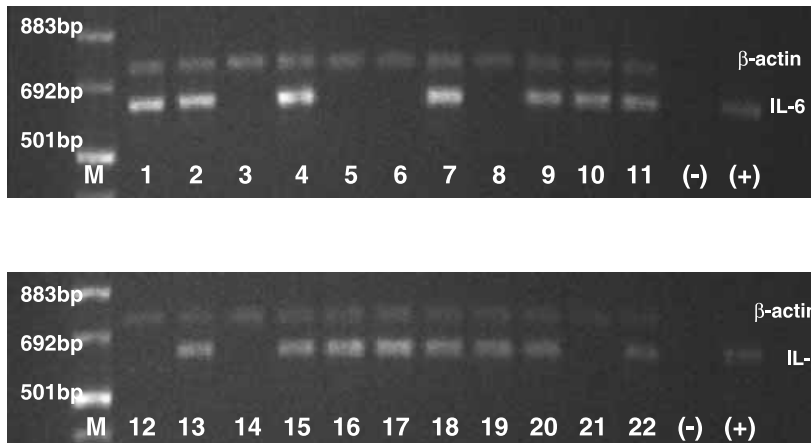PMBC obtained from postmenopausal women were incubated for 24 h with different concentrations of

Figure 3 RT-PCR analysis of IL-6 gene expression in the non-stimulated PBMC of postmenopausal women. RNA was isolated from the non-stimulated PBMC of the 22 postmenopausal women. cDNA was then synthesized and used as a template in a 33 cycle PCR reaction employing oligonucleotide primers specific for IL-6 and β-actin. PCR products were analysed by agarose gel electrophoresis stained with ethidium bromide and visualized under UV light. Lanes 1 to 22 correspond to the patients' numbers. M is the molecular marker pUC Mix Marker 8 (MBI; Fermentas AB, Vilnius, Lithuania). ($-$) is the negative control and (+) is the positive control.
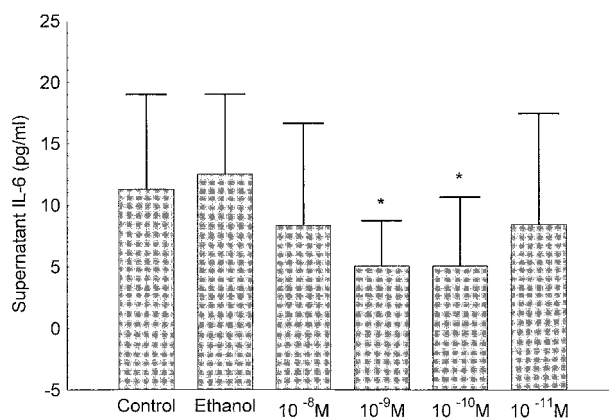
Figure 5 *In vitro* effects of 17β-oestradiol on the spontaneous IL-6 production by the PBMC of postmenopausal women. PBMC isolated from postmenopausal women ($n=22$) were incubated for 24 h with different concentrations of 17β-oestradiol. After the incubation period, the bioactive levels of IL-6 were measured in the supernatants using the B9 hybrydoma proliferation assay. 17β-Oestradiol at the concentrations of 10^{-9} M and 10^{-10} M significantly decreased the spontaneous IL-6 production into the culture supernatants by the PBMC of postmenopausal women (*$P<0.05$).

Figure 6 *In vivo* effects of 17β-oestradiol on the spontaneous IL-6 production by the PBMC of postmenopausal women. Seventeen out of twenty-two postmenopausal women were treated with 50 µg 17β-oestradiol/day transdermally (Estraderm 50MX). After 6 and 12 months of treatment, PMBC were isolated from venous blood and incubated for 24 h in a medium supplemented with FCS in a humidified atmosphere with 5% CO_2. After the incubation period, the bioactive levels of IL-6 were measured in the supernatants using the B9 hybrydoma proliferation assay. PBMC of postmenopausal women treated with 17β-oestradiol released significantly smaller amounts of bioactive IL-6 into the culture media after 12 months of treatment than at baseline (11.11 ± 4.44 pg/ml vs 14.83 ± 6.37 pg/ml (*$P<0.05$).

17β-oestradiol (10^{-8} to 10^{-11} M). After the incubation period, the culture supernatants were collected and the bioactive levels of IL-6 were measured. 17β–Oestradiol at the concentrations of 10^{-9} and 10^{-10} M significantly decreased the spontaneous IL-6 production by the PBMC of postmenopausal women ($P<0.05$) (Fig. 5).

In vivo effects of 17β-estradiol on spontaneous IL-6 production by the PBMC of postmenopausal women

Seventeen out of twenty-two postmenopausal women who were enrolled into the study were treated with 50 µg 17β-estradiol/day transdermally (Estraderm 50MX). After 6 and 12 months of the therapy, PMBC were isolated from venous blood and incubated for 24 h in a medium supplemented with FCS in a humidified atmosphere with 5% CO_2. After the incubation period, the levels of IL-6 were measured in the supernatants. PBMC of postmenopausal women after 12 months of oestrogen treatment released significantly smaller amounts of bioactive IL-6 into the culture media than at baseline (11.11 ± 4.44 pg/ml vs 14.83 ± 6.37 pg/ml, $P<0.05$) (Fig. 6).

Discussion

The aim of this study was to clarify whether oestrogen deprivation after menopause results in an enhanced IL-6 production by the PBMC of postmenopausal women. We found that serum levels of IL-6 in the postmenopausal 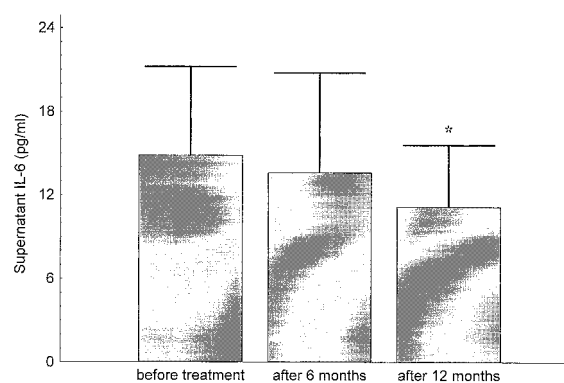women were higher compared with the young eugonadal female controls. The spontaneous IL-6 production by non-stimulated PBMC into the culture supernatants was also significantly higher in the postmenopausal women in relation to the young. We also found that, among the majority of postmenopausal women, IL-6 gene expression was present in the non-stimulated PBMC isolated directly from venous blood, which suggests the possibility of an endogenous activation of these cells *in vivo*. In addition, women with constitutive IL-6 gene expression in the non-stimulated PBMC had significantly lower serum 17β–oestradiol levels compared with those who did not express the IL-6 gene in the PBMC.

It has been pointed out that the enhanced IL-6 production by PBMC from postmenopausal women may not only be the consequence of the fall in oestrogen levels but may equally be determined by any of the many other factors which contribute to the aging process (Horan & Fox 1984, Winchurch *et al.* 1982, Ershler *et al.* 1993, Fagiolo *et al.* 1993, Wei *et al.* 1993, Belmin *et al.* 1995, Meyer *et al.* 1996, Myśliwska *et al.* 1998). To address these issues we also studied the *in vitro* as well as the *in vivo* effects of 17β-oestradiol on the spontaneous IL-6 production by the PBMC of postmenopausal women. Our *in vitro* experiments showed that 17β-oestradiol at the concentrations of 10^{-9} and 10^{-10} M significantly decreased the spontaneous IL-6 production by the PBMC of postmenopausal women. The *in vivo* treatment with 17β–oestradiol also resulted in a significant decrease in the spontaneous IL-6 production by the PBMC of the postmenopausal women after 12 months of therapy. From these results we can

conclude that oestrogen deprivation after menopause may contribute to the enhanced IL-6 production by the non-stimulated PBMC of the postmenopausal women.

Inappropriate expression and production of IL-6 is thought to be involved in the pathogenesis of numerous diseases, including osteoporosis (Manolagas & Jilka 1995), CHD (Huber *et al.* 1999) and AD (Mrak *et al.* 1995). Conversely, these disorders belong to the late complications of menopause and oestrogen replacement therapy may improve them (Lindsay *et al.* 1984, Stampfer & Colditz 1991, Tang *et al.* 1996).

Osteoporosis is among the most common causes of morbidity in older people (Chrischilles *et al.* 1991). IL-6 is a potent regulator of bone resorption (Ishimi *et al.* 1990). It is produced locally in the bone microenvironment, where it stimulates the differentiation and proliferation of haematopoietic osteoclast progenitor cells (Kurihara *et al.* 1991, Tamura *et al.* 1993). IL-6 seems to play a significant role in the pathogenesis of postmenopausal osteoporosis (Manolagas & Jilka 1995). In rodent models, IL-6-mediated stimulation of osteoclast differentiation and proliferation and its upregulation in oestrogen–depleted states is well documented (Girasole *et al.* 1992, Passeri *et al.* 1993). Bone marrow cells from women who had discontinued oestrogen replacement therapy within 1 month before marrow aspiration secreted significantly more IL-6 than bone marrow cells from either premenopausal or late postmenopausal subjects (Bismar *et al.* 1995). However, elevated plasma levels of IL-6 in postmenopausal women do not correlate with bone density or osteocalcin, a marker of bone turnover (Kania *et al.* 1995). However, epidemiological data show that serum IL-6 is a predictor of postmenopausal bone loss, and that the effect appears to be most relevant through the first postmenopausal decade (Scheidt-Nave *et al.* 2001).

CHD is the leading cause of death in women. It is relatively uncommon in premenopausal women but the increased incidence of the disease is seen with the loss of ovarian function (Stampfer *et al.* 1990). With the recognition that atherosclerosis is an inflammatory process (Ross 1999), several plasma markers of inflammation have been evaluated as potential tools for prediction of the risk of coronary events. Elevated plasma fibrinogen is now well established as an independent vascular risk factor (Cook & Ubben 1987, Ernst & Resch 1993). Fibrinogen is the chief determinant of plasma viscosity and plays a key role in platelet aggregation (Meade *et al.* 1985). Plasma C-reactive protein (CRP) has also recently been indicted as a strong cardiovascular risk factor (Ridker *et al.* 2000a). The possibility that CRP might, in fact, be a mediator of this risk is suggested by reports that CRP promotes the expression of tissue factor (thromboplastin) by monocytes (Cermak *et al.* 1993). Fibrinogen and CRP are acute-phase proteins, and the most potent and broadly effective stimulant of their production by human hepatocytes appears to be IL-6 (Castell *et al.* 1990). Therefore, in a prospective study in apparently healthy men, elevated levels of IL-6 were associated with increased risk of future myocardial infarction (Ridker *et al.* 2000b). Also, Volpato *et al.* (2001) showed that serum IL-6 levels are helpful in identifying a subgroup of older CHD female patients with a high risk of death over a period of 3 years.

An enhanced IL-6 production also seems to play a great role in the pathogenesis of AD − a chronic, low-grade, self-reinforcing acute-phase inflammatory process, confined to the affected regions of the brain (Mrak *et al.* 1995). A pathogenic role for IL-6 specifically is suggested by reports that IL-6 can be detected in early stage 'diffuse' plaques, solely in the brains of patients with AD (Hüll *et al.* 1996). Brain-specific over-expression of IL-6 in transgenic mice also results in age-related neurodegeneration associated with learning disabilities (Heyser *et al.* 1997). How this process results in the amyloid deposition, neuro-fibrillary tangles and loss of synapses and neurones characteristic of advanced plaques is not yet clear. Ganter *et al.* (1991) suggested that IL-6-mediated induction of acute-phase protease inhibitors (perhaps α2-macroglobulin) may prevent the normal proteolytic processing of the amyloid precursor protein, resulting in the formation and deposition of the insoluble β-amyloid protein that may be largely responsible for neurotoxicity. Our postmenopausal women did not have any signs of dementia or loss of cognitive functions. However, it would be reasonable to follow-up their health status until later decades of life in order to see if there is a link between an exaggerated IL-6 production after menopause and the development of AD.

In summary, our results indicate that oestrogen deprivation after menopause may enhance IL-6 production by the PBMC of postmenopausal women. It is therefore justifiable to end the speculations about the effects of oestrogen withdrawal on IL-6 production by immune cells. Further studies are warranted to clarify if the favourable effects of HRT are mediated by the repression of IL-6 production − the cytokine which seems to be a central mediator in the pathogenesis of the late complications of menopause.

Acknowledgements

References

Aarden LA, DeGroot ER, Schaap OL & Lansdrop PJ 1987 Production of hybrydoma growth factor by human monocytes. *European Journal of Immunology* **17** 1411–1416.

Belmin J, Bernard C, Corman B, Merval R, Esposito B & Tedgui A 1995 Increased production of tumor necrosis factor and interleukin-6 by arterial wall of aged rats. *American Journal of Physiology* **268** H2288–H2293.

Bismar H, Diel I, Ziegler R & Pfeilschifter J 1995 Increased cytokine secretion by human bone marrow cells after menopause or discontinuation of estrogen replacement. *Journal of Clinical Endocrinology and Metabolism* **80** 3351–3355.

Castell JV, Gomez-Lechon MJ, David M, Fabra R, Trullenque R & Heinrich PC 1990 Acute-phase response of human hepatocytes: regulation of acute-phase protein synthesis by interleukin-6. *Hepatology* **12** 1179–1186.

Cermak J, Key NS, Bach RR, Balla J, Jacob HS & Vercellotti GM 1993 C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. *Blood* **82** 513–520.

Chaudhary LR, Spelsbert TC & Riggs BL 1992 Production of various cytokines by normal human osteoblast-like cells in response to interleukin-1 beta and tumor necrosis factor alpha: lack of regulation by 17 beta estradiol. *Endocrinology* **130** 2528–2534.

Chrischilles EA, Butler CD, Davis CS & Wallace RB 1991 A model of lifetime osteoporosis impact. *Archives of Internal Medicine* **151** 2026–2032.

Cook NS & Ubben D 1987 Fibrinogen as a major risk factor in cardiovascular disease. *Trends in Pharmacological Sciences* **11** 444–451.

Ernst E & Resch KL 1993 Fibrinogen and viscosity as risk factors for subsequent cardiovascular events in stroke survivors. *Annals of Internal Medicine* **118** 956–963.

Ershler WB, Sun WH, Binkley N, Gravenstein S, Volk MJ, Kamoske G, Klopp RG, Roecker EB, Daynes RA & Weindruch R 1993 Interleukin-6 and aging: Blood levels and mononuclear cell production increase with advancing age and *in vitro* production is modifiable by dietary restriction. *Lymphokine and Cytokine Research* **12** 225–230.

Fagiolo U, Cossarizza A, Scala E, Fanales-Belasio E, Ortolani C, Cozzi E, Monti D, Franceschi C & Paganelli R 1993 Increased cytokine production in mononuclear cells of healthy elderly people. *European Journal of Immunology* **23** 2375–2378.

Ganter U, Strauss S, Jonas U, Weidemann A, Beyreuther K, Volk B, Berger M & Bauer J 1991 Alpha 2-macroglobulin synthesis in interleukin-6-stimulated human neuronal (SH-SY5Y neuroblastoma) cells. Potential significance for the processing of Alzheimer beta-amyloid precursor protein. *FEBS Letters* **282** 127–131.

Girasole G, Jilka RL, Passeri G, Boswell S, Boder G & Williams DC 1992 17 Beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts *in vitro*: a potential mechanism for the antiosteoporotic effect of estrogens. *Journal of Clinical Investigation* **89** 883–891.

Heyser CJ, Masliah E, Samimi A, Campbell IL & Gold LH 1997 Progressive decline in avoidance learning paralleled by inflammatory neurodegeneration in transgenic mice expressing interleukin 6 in the brain. *PNAS* **94** 1500–1505.

Horan MA & Fox RA 1984 Ageing and immune response: a unifying hypothesis. *Mechanisms of Ageing and Development* **26** 65–181.

Huber SA, Sakkinen P, Conze D, Hardin N & Tracy R 1999 Interleukin-6 exacerbates early atherosclerosis in mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* **19** 2364–2367.

Hüll M, Berger M, Volk B & Bauer J 1996 Occurrence of interleukin-6 in cortical plaques of Alzheimer's disease patients may precede transformation of diffuse into neuritic plaques. *Annals of the New York Academy of Science* **777** 205–212.

Ishimi Y, Miyaura C, Jin CH, Akatsu T, Abe E, Nakamura Y, Yamaguchi A, Yoshiki S, Matsuda T & Hirano T 1990 IL-6 is produced by osteoblasts and induces bone resorption. *Journal of Immunology* **145** 3297–3303.

Kania DM, Binkley N, Checovich M, Havighurst T, Schilling M & Ershler WB 1995 Elevated plasma levels of interleukin-6 in postmenopausal women do not correlate with bone density. *Journal of the American Geriatrics Society* **43** 236–239.

Kassem M, Harris SA, Spelsberg TC & Riggs BL 1996a Estrogen inhibits interleukin-6 production and gene expression in a human osteoblastic cell line with high levels of estrogen receptors. *Journal of Bone and Mineral Research* **11** 193–199.

Kassem M, Khosla S, Spelsberg TC & Riggs BL 1996b Cytokine production in the bone marrow microenvironment: failure to demonstrate estrogen regulation in early postmenopausal women. *Journal of Clinical Endocrinology and Metabolism* **81** 513–518.

Kurihara N, Civin C & Roodman GD 1991 Osteotropic factor responsiveness of highly purified populations of early and late precursors for human multinucleated cells expressing the osteoclast phenotype. *Journal of Bone and Mineral Research* **6** 257–261.

Lindsay R, Hart DM & Clark DM 1984 The minimum effective dose of estrogen for prevention of postmenopausal bone loss. *Obstetrics and Gynecology* **63** 759–763.

McKane WR, Khosla S, Peterson JM, Egan K & Riggs BL 1994 Circulating levels of cytokine that modulate bone resorption: effects of age and menopause in women. *Journal of Bone and Mineral Research* **9** 1313–1318.

Manolagas SC & Jilka RL 1995 Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. *New England Journal of Medicine* **332** 305–311.

Meade TW, Vickers MV, Thompson SG & Seghatchian MJ 1985 The effect of physiological levels of fibrinogen on platelet aggregation. *Thrombosis Research* **38** 527–534.

Meyer KC, Ershler W, Rosenthal NS, Lu XG & Peterson K 1996 Immune dysregulation in the aging human lung. *American Journal of Respiratory and Critical Care Medicine* **153** 1072–1079.

Mrak RE, Sheng JG & Griffin WST 1995 Glial cytokines in Alzheimer's disease. *Human Pathology* **26** 816–823.

Myśliwska J, Bryl E, Zorena K, Foerster J & Myśliwski A 1998 Increase of interleukin-6 and decrease of interleukin-2 production during the ageing process are influenced by the health status. *Mechanisms of Ageing and Development* **100** 313–328.

Papanicolaou DA & Vgontzas AN 2000 Interleukin-6: the endocrine cytokine. *Journal of Clinical Endocrinology and Metabolism* **85** 1331–1332.

Passeri G, Girasole G, Jilka RL & Manolagas SC 1993 Increased interleukin-6 production by murine bone marrow and bone cells after estrogen withdrawal. *Endocrinology* **133** 822–828.

Passeri G, Girasole G, Manolagas SC & Jilka RL 1994 Endogenous production of tumor necrosis factor by primary cultures of murine calvarial cells: influence on IL-6 production and osteoclast development. *Bone and Mineral* **24** 109–126.

Rickard D, Russell G & Gowen M 1992 Oestradiol inhibits the release of tumor necrosis factor but not interleukin-6 from adult human osteoblasts *in vitro*. *Osteoporosis International* **2** 94–102.

Ridker PM, Hennekens CH, Buring JE & Rifai N 2000a C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *New England Journal of Medicine* **342** 836–843.

Ridker PM, Rifai N, Stampfer MJ & Hennekens CH 2000b Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation* **101** 1767–1772.

Rifas L, Kenney JS, Marcelli M, Pacifici R, Cheng SL, Dawson LL & Avioli LV 1995 Production of interleukin-6 in human osteoblasts and human bone marrow stromal cells: evidence that induction by interleukin-1 and tumor necrosis factor-alpha is not regulated by ovarian steroids. *Endocrinology* **136** 4056–4067.

Ross R 1999 Atherosclerosis – an inflammatory disease. *New England Journal of Medicine* **340** 115–126.

Scheidt-Nave C, Bismar H, Leidig-Bruckner G, Woitge H, Seibel MJ, Ziegler R & Pfeilschifter J 2001 Serum interleukin 6 is a major predictor of bone loss in women specific to the first decade past menopause. *Journal of Clinical Endocrinology and Metabolism* **86** 2032–2042.

Stampfer MJ & Colditz GA 1991 Estrogen replacement therapy and coronary heart disease: a qualitive assessment of the epidemiologic evidence. *Preventive Medicine* **20** 47–63.

Stampfer MJ, Colditz GA & Willett WC 1990 Menopause and heart disease. A review. *Annals of the New York Academy of Science* **592** 193–203.

Straub RH, Hense HW, Andus T, Scholmerich J, Riegger GA & Schunkert H 2000 Hormone replacement therapy and interrelation between serum interleukin-6 and body mass index in postmenopausal women: a population based study. *Journal of Clinical Endocrinology and Metabolism* **85** 1340–1344.

Tamura T, Udagawa N, Takahashi N, Miyaura C, Tanaka S, Yamada Y, Koishihara Y, Ohsugi Y, Kumaki K & Taga T 1993 Soluble interleukin-6 receptor triggers osteoclast formation by interleukin-6. *PNAS* **90** 11924–11928.

Tang MX, Jacobs D, Stern Y, Marder K, Schofield P, Gurland B, Andrews H & Mayeux R 1996 Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. *Lancet* **348** 429–432.

Volpato S, Guralnik JM, Ferrucci L, Balfour J, Chaves P, Fried LP & Harris TB 2001 Cardiovascular disease, interleukin-6, and risk of mortality in older women: the women's health and aging study. *Circulation* **103** 947–953.

Wei J, Xu H, Davies JL & Hemmings GP 1993 Increase of plasma IL-6 concentration with age in healthy subjects. *Life Sciences* **51** 1953–1956.

Winchurch RA, Birmingham W, Hilberg C & Munster A 1982 Effect of endotoxin on immunity in aging mice. *Cell Immunology* **67** 384–389.