EFFECTS OF MEDROXYPROGESTERONE ACETATE AND β-ESTRADIOL ON INTERLEUKIN-6 PRODUCTION FROM OSTEOBLASTS AND BONE MARROW MACROPHAGES OF WISTAR RATS OF DIFFERENT AGES

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ABSTRACT—Using 18-week-old and 52-week-old Wistar rats, we examined interleukin-6 (IL-6) production from osteoblasts and bone marrow macrophages treated with medroxyprogesterone acetate (MPA) and/or β-estradiol. The level of IL-6 production by osteoblasts was increased by treatment with MPA and, reversely, decreased by treatment with β-estradiol. These changes were especially remarkable in osteoblasts of 52-week-old rats. Additionally, and in contrast, the production of IL-6 by bone marrow macrophages was not significantly changed by treatment with both agents. These data suggest that because the increased production of IL-6 by osteoblasts treated with MPA in opposition with β-estradiol, MPA should be careful for osteoporosis dependent upon osteoclasts activated by IL-6. Finally, there was a marked difference in the amount of IL-6 produced between osteoblasts and bone marrow macrophages.

KEY WORDS: Bone marrow macrophage, β-Estradiol, Interleukin-6, Medroxyprogesterone acetate, Osteoblast, Rat.

INTRODUCTION

Recently several reports have described a relationship between cytokine production and bone remodeling or osteoporosis (Manolagas, 1994). Remodeling is carried out by osteoclasts and osteoblasts that exist in the bone matrix (Robey et al., 1985). During the last few years, interleukin-6 (IL-6) has gained prominence among the cytokines that are relevant to bone metabolism (Hughes et al., 1993). On the other hand, it is also known that IL-6 is important in hematopoiesis and immunity (Mei et al., 1991). We have been studying the cytokine level in bone marrow associated with aging. In that study, we found that the IL-6 level in the bone marrow of 52-week-old rats was decreased compared with that of 18-week-old ones (Futamura et al., 1993). IL-6 also stimulates osteoclasts, which is followed by bone resorption, and activates the bone resorption process cooperatively with IL-1 in vivo and in vitro (Ishimi et al., 1990). Further, IL-6 seems to play a causative role in the pathologic bone resorption associated with multiple myeloma (Klein et al., 1991), Paget’s disease (Roodman et al., 1992), and postmenopausal osteoporosis (Jilka et al., 1992).

Manolagas et al. (1993) described that the absence of estrogen caused an increase in IL-6 production.
production in the bone marrow followed by bone resorption. Medroxyprogesterone acetate (MPA), which is used for therapy of breast cancer, possesses anti-estrogen action and strong progesterone action (Fujimoto et al., 1989). In this study, we examined the effects of MPA and \( \beta \)-estradiol on IL-6 production from osteoblasts and bone marrow macrophages using 18 and 52 week-old Wistar rats, as well as the difference between osteoblasts and bone marrow macrophages with respect to IL-6 production.

MATERIALS AND METHODS

Test material: MPA and \( \beta \)-estradiol were purchased from Sigma (USA), and dimethylsulfoxide (DMSO) was purchased from Wako Pure Chemical Inc. MPA and \( \beta \)-estradiol were dissolved and diluted in DMSO. Standard IL-6 was obtained from Intergen Co., Ltd. (USA). Other reagents used in this study were special grade.

Animals: Thirty six female Slc: Wistar rats (SPF), 17 and 51 weeks of age, were obtained from Japan SLC Inc. (Shizuoka, Japan). The animals were housed in stainless steel cages in a barrier system at 23±2°C and humidity of 55±15% with twelve hours of light per day (7 a.m. – 7 p.m.), and fed pellet food (FR-2, Funabashi Farm Co., Ltd., Japan) and tap water ad libitum. These animals were maintained under these conditions for 1 week and then used for preparation of osteoblasts and bone marrow macrophages. All procedures on the animals conformed to the Guide for the Regulation of Animal Experimentation (Japanese Association for Laboratory Animal Science, 1987).

Isolation of bone marrow macrophages: Bone marrow macrophages were isolated by the adhesion method (Sunada et al., 1985; Stephens et al., 1988; Kukita et al., 1992). Briefly stated, each rat was anesthetized by intraperitoneal injection with sodium pentobarbital at a dose of 25 mg/kg. After the animals had been exsanguinated, their femur bones were removed, and the bone marrow was withdrawn and suspended in sodium phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 400×g for 5 minutes at 4°C. The cell pellet was mixed with 5 ml of PBS and layered onto a Ficoll solution (Pharmacia Biotech), that was then centrifuged at 3000×g for 30 minutes at 15°C, and the mononuclear cells were obtained from the middle layer. These cells were then washed with PBS twice, suspended in culture medium (AIV-M, GIBCO), inoculated into PRIMARIA™ tissue culture dishes (Becton Dickinson) previously coated with PBS containing 10% fetal bovine serum (Dainippon Pharmaceutical Co., Ltd.) and incubated for 1 hour at 37°C under a 5% \( CO_2 \)/95% air atmosphere. After each dish had been washed with culture medium to remove the non-adhering cells, medium supplemented with 0.5% EDTA was added to the dish to release the adherent cells, i.e., bone marrow macrophages. These cells were washed three times with the same medium, and the final suspension was adjusted to a concentration of 1×10^5 cells/ml.

Isolation of osteoblasts: Osteoblasts were isolated from femur bone from which the bone marrow had been removed according to the method previously described (Ernst et al., 1988; Johansen et al., 1992). Briefly stated, the cut femur bone was shaved with a knife and the bone obtained fragments were collected into a tube. These fragments were digested with 1% collagenase (Nitta Gelatin Co. Ltd.) in PBS for 20 minutes at 37°C. After the tube had been agitated, the resulting cell suspension was filtered through a nylon-mesh filter. The filtered cells were washed twice with culture medium consisting of MEM medium (Nissui Pharmaceutical Co. Ltd.) supplemented with 10% charcoal-treated FBS (JRH Bioscience). The cells were then inoculated into plastic tissue culture dishes (FALCON) and incubated at 37°C under 5% \( CO_2 \)/95% air atmosphere for 24 hours. After each dish had been washed with PBS to remove the non-adhering cells, medium supplemented with 5% EDTA was added to the plate to release the adherent cells, i.e., osteoblasts. These cells were washed three times with the same medium, and the final suspension was adjusted to a concentration of 1×10^5 cells/ml. Cells in the bone cell culture expressing alkaline phosphatase, an osteoblastic phenotype, were identified by staining for this enzyme. The reaction mixture of naphthol AS-MX phosphate (Sigma) and fast blue BB conjugate (Sigma) in 0.1 M Tris-HCl buffer (pH 9.2) was filtered directly onto a slide.
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**Statistical analysis**: Results were expressed as mean±S.E. of nine animals. Statistical analysis of the data were carried out by analysis of variance and Dunnet's test. A difference from the respective control at the P<0.05 level was regarded as statistically significant.

RESULTS

**Effects of MPA and β-estradiol on osteoblasts** (Figs. 1 and 2): In the presence and absence of β-estradiol, the level of IL-6 produced by osteoblasts from 18-week-old rats was significantly increased by treatment with 1 and 10 μM MPA. In the case of the 52-week-old rats, their osteoblasts treated with 0.1, 1 and 10 μM MPA produced significant high level of IL-6 regardless of the presence or absence of β-estradiol. A significant dose-dependent decrease in IL-6 production in osteoblasts of 18 and 52-week-old rats was observed with 0.01 nM or more β-estradiol treatment in spite of the addition of MPA. Especially, a remarkable decrease was seen in...
52-week-old rat cells under 1 nM β-estradiol addition. Additionally, the content of IL-6 was increased in the medium of vehicle-treated osteoblasts from 52-week-old rats compared with that of 18-week-old ones.

**Effects of MPA and β-estradiol on bone marrow macrophages (Figs. 3 and 4):** The content of IL-6 in bone marrow macrophage cultures was not significantly different between each drug-treatment group.

These results were inconsistent with those on osteoblasts. The vehicle-treated bone marrow macrophages from 18-week-old rats produced 27.0 pg/ml of IL-6; and those from 52-week-old animals, 17.5 pg/ml (35% decrease).

**DISCUSSION**

It was reported that MPA possesses strong progesteronic activity (20 to 50 fold-higher than progesterone) and no estrogentic action (Poulin et al., 1989). MPA is a useful drug for therapy of breast cancer in spite of several adverse reaction...
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it produces, i.e., lipidosis and thrombosis (Racca et al., 1989). But toxicity regarding osteogenesis and osteoporosis has been discussed in few reports.

Recently it was indicated that several cytokines, i.e., IL-1 and IL-6, are related to the osteoporosis in menopause and estrogen therapy (Morimoto et al., 1993). Girasole et al. (1992) reported about the effects of \( \beta \)-estradiol on IL-6 synthesis in non transformed osteoblastic cell lines from mice and rats: IL-6 production was stimulated as much as 10,000-fold in response to the combination of recombinant IL-1 and tumor necrosis factor \( \alpha \), and \( \beta \)-estradiol decreased the level of IL-6 production. Another study indicated that estrogen loss resulted in an IL-6-mediated stimulation of osteoclastogenesis, which suggests a possible mechanism for increased bone resorption in postmenopausal osteoporosis (Jilka et al., 1992). Passeri et al. (1993) reported that osteoblasts from ovariec-toximized mice produced IL-6 in the absence of \( \beta \)-estradiol.

In this study, we observed an increase in IL-6 production by osteoblasts treated with MPA and a decrease in it by those treated with \( \beta \)-estradiol. These changes due to MPA treatment were not reported previously. Our data indicate that MPA induced IL-6 production from osteoblasts of aged rats. Such increased production would probably cause osteoclastogenesis in vivo. In fact, several researchers have reported that MPA might have an adverse effect on bone, i.e., loss of bone density, in clinical study (Cundy et al., 1991; Gallagher, et al., 1991; Kaunitz, et al., 1993). Therefore the results of our in vitro animal study also suggest that therapy with MPA should be used with caution in the case of osteoporosis and that \( \beta \)-estradiol may possibly improve the IL-6-mediated osteoclastogenesis in postmenopausal women.

In contrast, IL-6 production from bone marrow macrophages was not stimulated by MPA treatment and not changed by \( \beta \)-estradiol. Furthermore, the production of IL-6 by osteoblasts from 52-week-old rats was greater than that by the cells from 18-week-old ones, the reverse was the case for the bone marrow macrophages. These results indicate marked differences between bone marrow macrophages and osteoblasts, and differences in sensitivity on aged rats with respect to IL-6 production. This present study and our previous study (Futamura et al., 1993) allow the speculation that osteoclasts in bone are activated by IL-6 released from osteoblasts neighboring osteoclasts and not by IL-6 released from macrophages in the bone marrow.

In conclusion, the results described in this paper document the increased production of IL-6 by osteoblasts treated with MPA in opposition with \( \beta \)-estradiol treatment, and the marked difference between osteoblasts and bone marrow macrophages with respect to age dependency and drug/hormone sensitivity of IL-6 production.

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