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Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion

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Interleukin-6 (IL-6) is a multifunctional cytokine whose circulating levels are under physiological conditions below detection, but whose production is rapidly and strongly induced by several pathological and inflammatory stimuli. IL-6 has been implicated in a number of cell functions connected to immunity and hematopoiesis. Recently, it has been proposed to act as a stimulator of osteoclast formation and activity, in particular following estrogen depletion. The purpose of this study was to gain additional insights into the role of IL-6 during development, as well as in physiological and pathological conditions. We report here that IL-6 deficient mice generated by gene targeting are viable and do not present any evident phenotypic abnormality. However, analysis of bone metabolism revealed a specific bone phenotype. IL-6 deficient female mice have a normal amount of trabecular bone, but higher rates of bone turnover than control littermates. Estrogen deficiency induced by ovariectomy causes in wild type animals a significant loss of bone mass together with an increase in bone turnover rates. Strikingly, ovariectomy does not induce any change in either bone mass or bone remodeling rates in the IL-6 deficient mice. These findings indicate that IL-6 plays an important role in the local regulation of bone turnover and, at least in mice, appears to be essential for the bone loss caused by estrogen deficiency.

Key words: estrogens/gene targeting/interleukin-6/osteoporosis

Introduction

The skeleton is a dynamic organ in which mineralized bone is continously resorbed by osteoclasts, and new bone is formed by osteoblasts. This process, known as bone remodeling, is normally highly regulated with maintenance of a normal amount of bone (Parfitt *et al.*, 1988). Systemic hormones like parathyroid hormone and sex steroids are known to be important regulators of bone cell function, and alterations in their circulating levels can lead to abnormal bone metabolism (Raisz, 1988). In particular, estrogen deficiency caused by natural or surgically induced menopause results in increased bone turnover, with bone resorption exceeding bone formation. This imbalance leads to bone loss which predisposes to osteoporosis, a common disabling condition in post-menopausal age [reviewed by Raisz (1988) and Dempster and Lindsay (1993)]. The molecular mechanism for the protective action of estrogens on the skeleton is not fully understood. Estrogen receptors have been detected in osteoblast-like cells (Eriksen *et al.*, 1988; Komm *et al.*, 1988), suggesting that estrogens can act directly on bone cells, and recent studies suggest that they regulate the production of cytokines in the bone microenviroment (reviewed by Horowitz, 1993). These cytokines include the colony-stimulating factors, interleukin-1 (IL-1), and tumor necrosis factor α (TNFα). Additional evidence suggests that IL-6 may play an important role in mediating the effects of estrogens on bone: (i) IL-6 can stimulate the proliferation of early hematopoietic progenitor cells, from which osteoclastic cells take origin (Michalevicz *et al.*, 1989); (ii) osteoblast-like cell lines and murine bone marrow stromal cells secrete IL-6 in response to treatment with IL-1 and TNFα, and this production can be inhibited by 17β-estradiol (Girasole *et al.*, 1992); and (iii) anti-IL-6 monoclonal antibodies can inhibit the increase in osteoclast precursors occurring in estrogen depleted mice (Jilka *et al.*, 1992).

IL-6 also has a number of different activities on lymphohemopoietic tissues as well as on tissues of different origin. It induces B cell maturation, growth of myeloma/plasmacytoma cells and of T cells, and proliferation of mesangial cells and of thymocytes (reviewed by Kishimoto and Hirano, 1988). IL-6 is also a potent inducer of the acute-phase response in the liver (Gauldie *et al.*, 1987). Deregulated production of this cytokine has been implicated in the pathogenesis of several auto-immune diseases and of multiple myeloma (reviewed by Hirano *et al.*, 1990). Moreover, IL-6 overproduction was shown to trigger plasmacytoma formation in transgenic mice (Suematsu *et al.* 1992). It was recently shown that a number of structurally related cytokines, including leukemia inhibitory factor (LIF), oncostatin M (OncM), ciliary neurotrophic factor (CNTF) and interleukin-11 (IL-11), share with IL-6 the same effector molecule, gp130. This finding explains the partial functional overlap described among this family of molecules in a number of biological systems (reviewed by Taga and Kishimoto, 1992).

To study *in vivo* the role of this cytokine, IL-6 deficient (IL-6$^{-/-}$) mice were generated by gene targeting. We show that IL-6 deficiency in mice is compatible with normal survival and development, and that both mutant males and females are fertile. A comparative analysis of bone structure and metabolism in wild type and mutant mice demonstrates that IL-6 is indeed required for the generation of bone loss following estrogen withdrawal.

Results

Mutating the IL-6 gene in ES cells

To abolish IL-6 function, the mouse IL-6 gene was mutated by replacing a 2.1 kb fragment, containing the proximal promoter region and the first three exons of the gene, with an MC1-Neo poly(A)$^+$ cassette. This eliminated the sequences coding for the amino-terminal half of the protein, essential for biological activity (Brakenhoff *et al.*, 1989). The replacement vector shown in Figure 1A was then used to mutate the endogenous IL-6 gene in embryonic stem (ES) cells by homologous recombination, and the positive-negative selection method (Mansour *et al.*, 1988) was applied to enrich for homologous against random integration events. ES cell clones which had undergone the correct recombination event were initially identified by polymerase chain reaction (PCR) analysis, and subsequently confirmed by Southern blotting (Figures 1 and 2A). The probe 5′, shown in Figure 1C, was used to discriminate between wild type and mutant alleles (Figure 2A). To verify that the recombination event was correct, the same filters were stripped and hybridized with a fragment from the mouse IL-6 cDNA corresponding to exons I, II and III, which should be deleted in the mutant allele; as predicted, no hybridization to the mutant allele was detected (not shown). A probe corresponding to the *Neo* sequence was used to verify that only one copy of the vector had integrated in the genome (Figure 2A, NEO probe). The recombination boundary at the 3′ end of the homology was verified by digestion with *Sac*I and hybridization with the mouse IL-6 cDNA. As predicted, no difference in size was detected between the wild type and the mutant alleles, indicating that the recombination end point was correct (not shown).

In total, six mutant clones were isolated from 240 doubly resistant clones, after an enrichment of ~5-fold obtained with Gancyclovir. Following injection into recipient blastocysts, one clone (no. 120) gave rise to chimeras able to transmit the mutation through the germ line.

Generation of mice homozygous for the IL-6 mutation

Heterozygous animals were intercrossed to obtain mice homozygous for the mutant IL-6 allele. The genotypic analysis of the litter from one such intercross is shown in Figure 2B. The litters were normal in size, and genotypic analysis revealed that mice homozygous for the mutation (IL-6$^{-/-}$) were born at the expected Mendelian ratio of 25%. They appeared to be normal in size and developed normally. It has recently been shown that LIF deficient mouse females are sterile because of a specific defect in embryo implantation (Stewart *et al.*, 1992). In contrast, IL-6 deficient females were found to be fertile, indicating a degree of functional specificity between the two cytokines.

The IL-6 mutation is a null mutation

IL-6 is normally not present at detectable levels in the tissues and in the blood stream of healthy animals. To demonstrate that the mutant allele could not produce any functional IL-6, we used bacterial lipopolysaccaride (LPS), a strong inducer of IL-6 production. LPS was injected intraperitoneally into IL-6$^{-/-}$ mice and control littermates, and total RNA was extracted from the spleen of the same animals and analyzed by Northern blotting. As expected, the spleen of the untreated animals did not contain any detectable IL-6 mRNA (Figure 3, lanes 1 and 3). However, while LPS injection strongly induced IL-6 mRNA synthesis in the wild type mice,

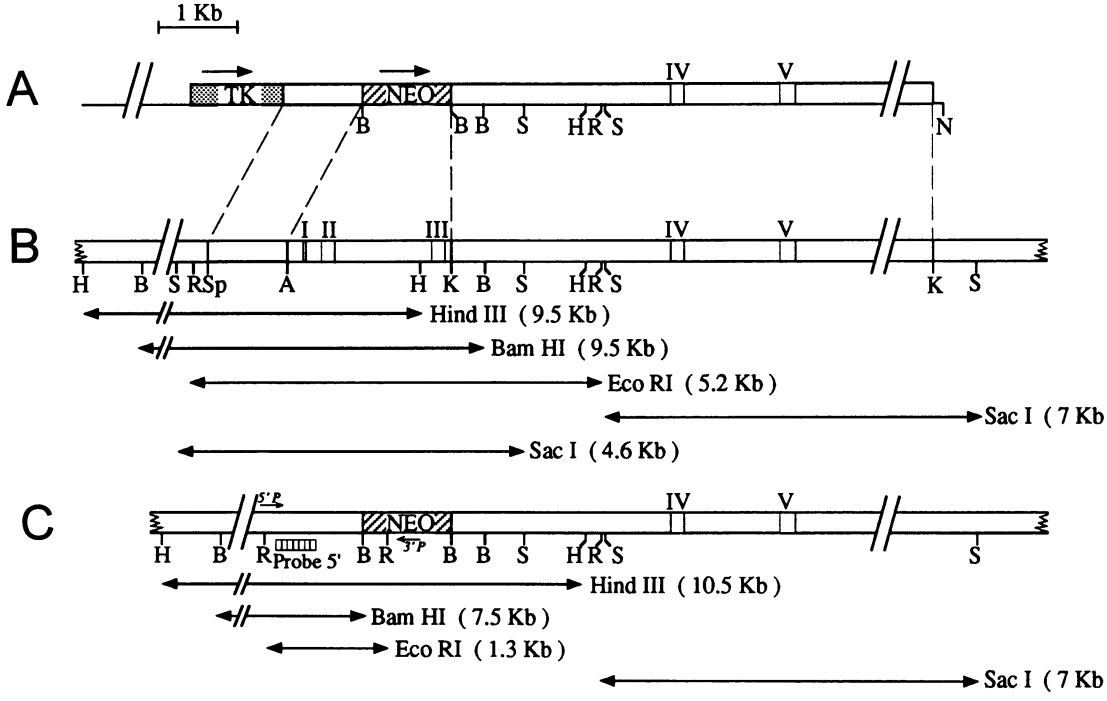

Fig. 1. Strategy used to mutate the endogenous IL-6 gene by homologous recombination. (**A**) Replacement vector used for gene targeting. The transcriptional orientation of the *Neo* and TK genes is indicated by arrows. (**B**) Structure of the wild type IL-6 allele; white boxes represent the five exons; only the restriction sites relevant to the construct and to the Southern blotting analysis are indicated: H, *Hin*dIII; B, *Bam*HI; R, *Eco*RI; Sp, *Spe*I; A, *Aat*II; K, *Kpn*I; S, *Sac*I. (**C**) Predicted structure of the mutated allele. The restriction fragments generated from the wild type and the mutant allele by digestion with *Hin*dIII, *Bam*HI, *Eco*RI and *Sac*I are shown. 5′P and 3′P: primers used for the diagnostic PCR reaction. Probe 5′: probe used for the Southern blotting analysis.

no signal could be detected in the IL-6 homozygous mutant mice (Figure 3, compare lanes 2 and 4).

To confirm this result at the protein level, the amount of IL-6 in the serum was measured with an ELISA assay. While IL-6 was detected in the serum of the LPS treated wild type mice at a concentration of 100 ng/ml, the protein was not detectable in the serum of the mutant mice (Figure 3). We also tested the same sera with a more sensitive assay, based on the growth promoting activity of IL-6 on the hybridoma cell line 7TD1 (Van Snick *et al.*, 1986). To our surprise, a weak but clear activity (~300 times lower than in the wild type mice) was detected in the serum of the mutant mice. However, this could not be inhibited by a neutralizing anti-murine IL-6 monoclonal antibody, which in contrast abolished an equivalent activity either from recombinant murine IL-6 or from wild type mouse serum (data not shown). This result confirmed that the mutant mice could not produce any functional IL-6. Interestingly, other two IL-6 dependent hybridoma cell lines, T1165 and B9, have been recently reported to be responsive to IL-11 (Paul *et al.*, 1990; Burger and Gramatzki, 1993). By analogy, it is reasonable to speculate that the growth promoting activity detected in our assay could be due to the presence of low amounts of IL-11 in the serum of the LPS treated mice.

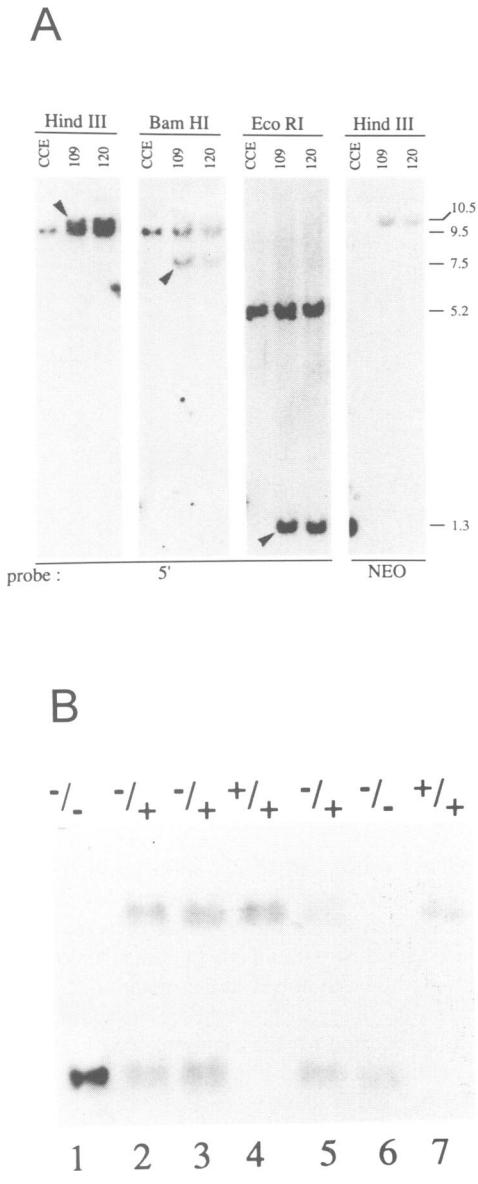

Fig. 2. Southern blotting analysis. (**A**) Genomic DNA from CCE wild type cells and the homologous recombinant clones, nos 109 and 120, was digested with the indicated restriction enzymes and analyzed by Southern blotting. Due to the presence of repetitive sequences both at the 5′ and at the 3′ end of the gene, it was not possible to use a probe corresponding to sequences external to the replacement vector. The 5′ probe shown in Figure 1C was used to discriminate between the wild type and mutant alleles. The size of the fragments obtained is indicated; arrowheads indicate the bands corresponding to the mutant allele. A probe corresponding to the *Neo* sequence was used to verify that only one copy of the vector had integrated in the genome (NEO probe). (**B**) Genotypic analysis of a 3 week old offspring from an intercross between two heterozygotes. The DNA was digested with *Eco*RI and hybridized with the 5′ probe. The genotypes are shown on top of the lanes; mice in lanes 1 and 6 are homozygous for the mutant IL-6 allele.

Bone volume and bone turnover in the mutant mice

In the light of several reports indicating that IL-6 plays a role in osteoclastogenesis (Ishimi *et al.*, 1990; Jilka *et al.* 1992; Ohsaki *et al.*, 1992) as well as in bone formation (Hughes and Howells, 1993), we decided to investigate the effects of IL-6 deficiency on bone structure and metabolism using quantitative histomorphometry. We analyzed bones obtained from IL-$6^{-/-}$ and IL-$6^{+/+}$ female mice, under both normal and estrogen depleted conditions. Briefly, 4 month old IL-$6^{-/-}$ females and wild type controls were ovariectomized, while corresponding littermates were left intact as controls. All mice were labeled *in vivo* by injection of oxytetracycline, followed 6 days later by injection of calcein. These fluorochrome labels allow for quantification of the amount of bone formed per day. 32 days after surgery the animals were sacrificed, and the cancellous bone of the distal femoral metaphysis was analyzed by static and dynamic

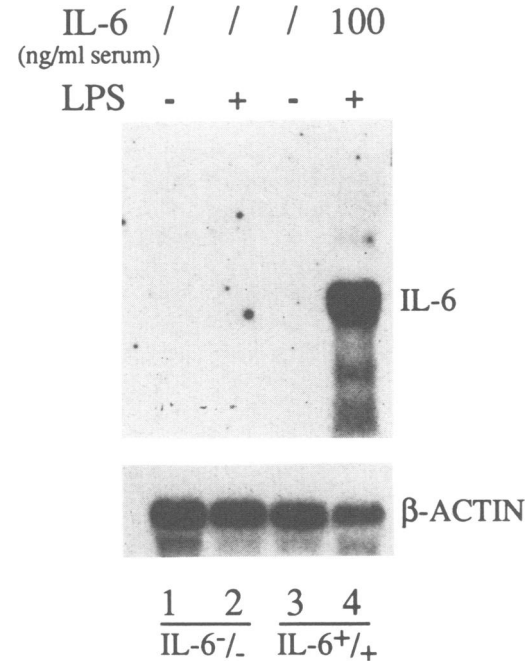

Fig. 3. Northern blot analysis of total RNA (20 μg) from the spleen of two IL-$6^{-/-}$ mice (lanes 1 and 2) and two IL-$6^{+/+}$ littermates (lanes 3 and 4), either injected with LPS to induce IL-6 production (+, lanes 2 and 4), or not injected (−, lanes 1 and 3). The signals detected with a murine IL-6 cDNA probe and with a β-actin internal control are shown. The amount of IL-6 detected by an ELISA assay in the serum of the same mice is shown on top of the lanes.

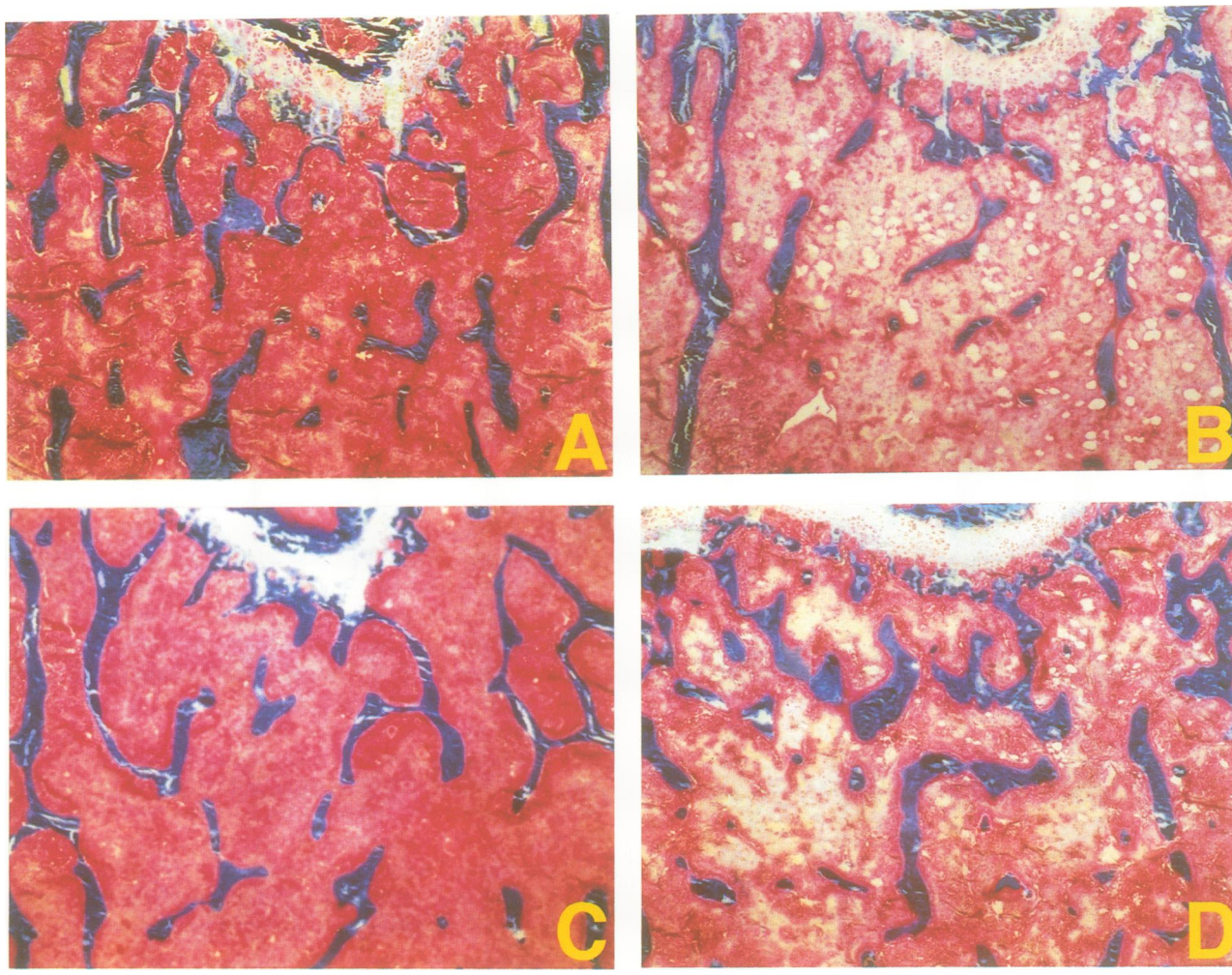

Fig. 4. Photomicrographs showing cancellous bone in the femoral metaphysis from 5 month old mice. (**A** and **B**) IL-6$^{+/+}$; (**C** and **D**) IL-6$^{-/-}$, non-ovariectomized (**A** and **C**) and ovariectomized (**B** and **D**). Longitudinal sections were stained with Masson's trichrome and photographed with a Nikon Microphot microscope. Bone trabeculae are stained in blue, while the bone marrow stroma is red.

histomorphometry. Figure 4 shows photomicrographs of sections from the distal femur illustrating trabecular bone, and Figure 5 shows indices of bone turnover and mineralization.

Regardless of their estrogen status, IL-6$^{-/-}$ mice had higher bone turnover than non-ovariectomized IL-6$^{+/+}$ littermates. In the non-ovariectomized animals the extent of the osteoid surface, which constitutes the newly synthesized bone matrix not yet mineralized, was 7.7% as compared with 3.5% in the wild type mice (Figure 5A) and the rate of bone formed per day was three times higher (Figure 5B). The mean width of the osteoid seams (Figure 5C) and the mean duration of the mineralization lag time (Figure 5D) were not different from those in the non-ovariectomized IL-6$^{+/+}$ mice, indicating normal mineralization processes. With respect to cellular parameters of bone remodeling, the extent of bone surface covered with active osteoblasts (Figure 5E) was only slightly higher, and the number of osteoclasts in resorption lacunae, an index of bone resorption, was not different from that in wild type mice (Figure 5F). Moreover, the mean number of osteoclasts identified by morphological criteria in the samples of bone examined was not different between mutant and wild type mice (not shown).

The accelerated bone turnover rate displayed by the IL-6$^{-/-}$ mice did not cause statistically significant changes in the amount of trabecular bone (Figures 6A and 4; compare panels A and C), indicating that the coupling between bone resorption and formation is maintained. In contrast, cortical bone volume was significantly lower in the mutant mice than in their wild type littermates (Figure 6B). These findings indicate that IL-6 is required for the maintenance of normal bone turnover in the trabecular bone, as well as for the development of a normal amount of cortical bone, but not for maintenance of trabecular bone mass.

IL-6$^{-/-}$ mice do not lose bone following estrogen depletion

As already shown for Swiss-Webster mice (Bain *et al.*, 1993), removal of the ovarian function in the IL-6$^{+/+}$ mice caused marked trabecular bone loss (Figure 4; compare panels A and B). Trabecular bone volume decreased by 50% (Figure 6A), mainly as a result of a decrease in trabecular density (not shown). Indices of bone turnover (Figure 5A–E) and of bone resorption (Figure 5F) increased in the range 3- to 5-fold. In contrast, no change was induced by ovariectomy in the IL-6$^{-/-}$ mice. The indices of bone turnover remained similar to the values of the non-ovariectomized animals, and no loss of bone was

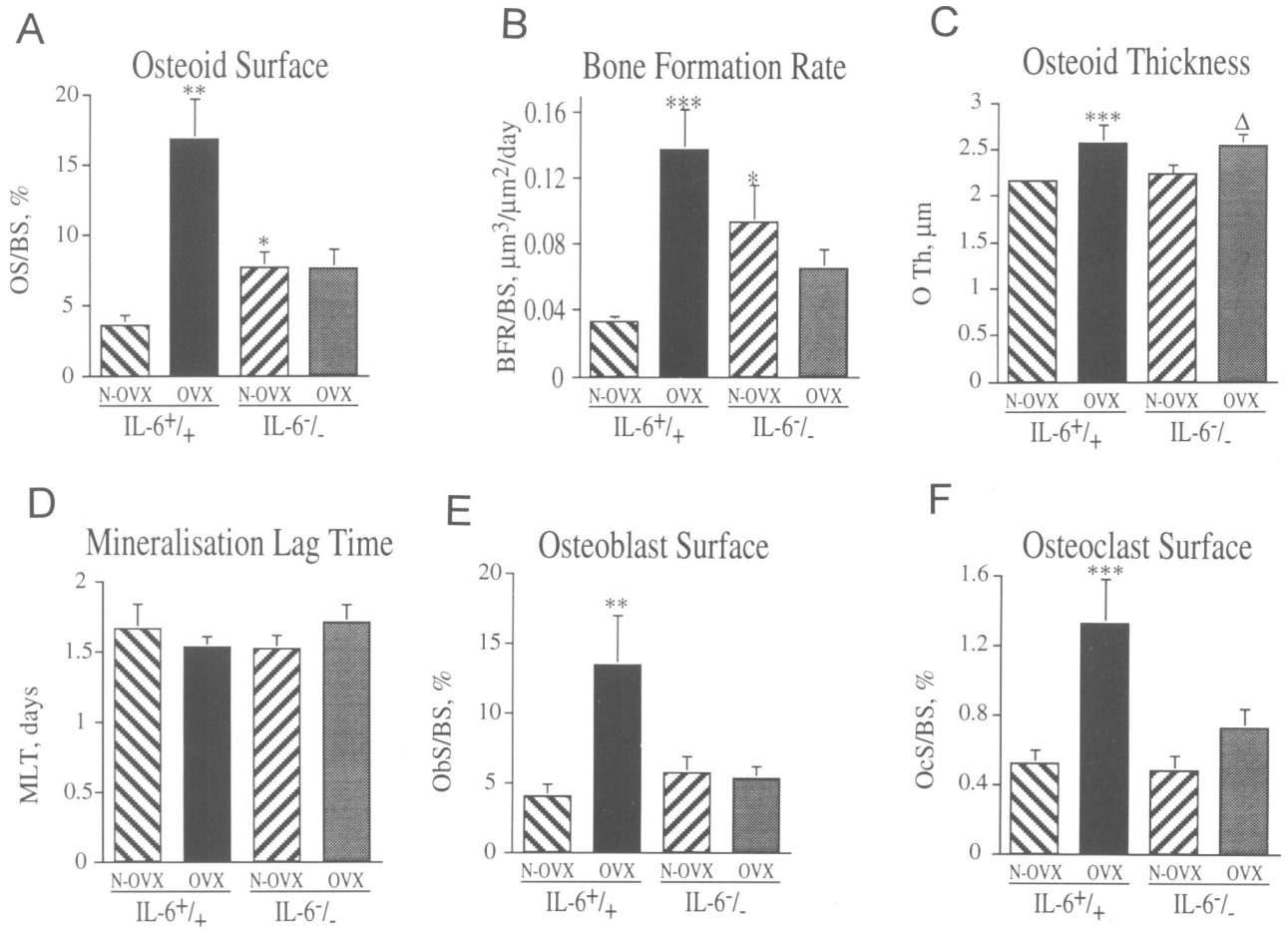

Fig. 5. Static and dynamic histomorphometric analysis in the femora from ovariectomized (OVX) and non-ovariectomized (N-OVX) IL-$6^{+/+}$ and IL-$6^{-/-}$ mice. Bars represent means ± SEM. The asterisks indicate a statistically significant difference from IL-$6^{+/+}$ N-OVX; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. (**A**) Osteoid surface, expressed as a percentage of the total trabecular bone surface (OS/BS, %). (**B**) Bone formation rate per unit of trabecular bone surface (BFR/BS, $\mu m^3/\mu m^2$/day). (**C**) Osteoid thickness (OTh, μm); Δ, significantly different from IL-$6^{-/-}$ N-OVX, $P < 0.05$. (**D**) Mineralization lag time (MLT/day). (**E**) Osteoblast surface, expressed as a percentage of the total trabecular surface (ObS/BS, %). (**F**) Osteoclast surface, expressed per unit of mineralized trabecular bone surface (Ocs/MdBS, %).

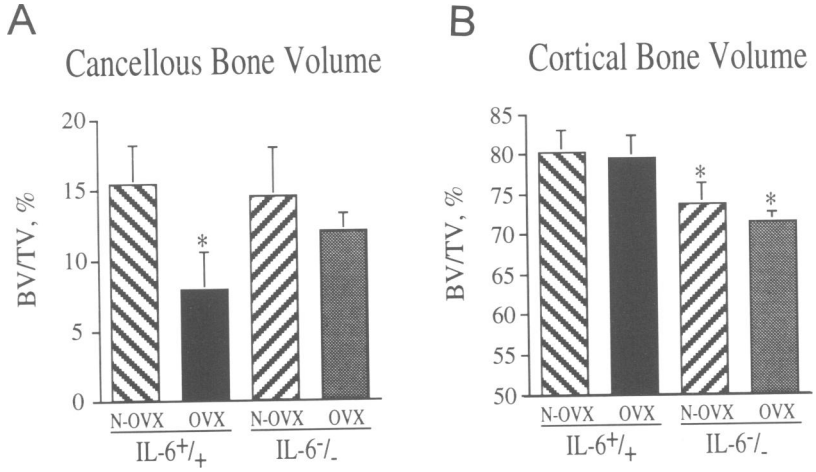

Fig. 6. Bone volume measurements. (**A**) Cancellous bone volume (BV/TV, %), expressed as the percentage of the metaphyseal area occupied by cancellous bone excluding the cortices. (**B**) Cortical bone volume (BV/TV, %), expressed as the percentage of the cortical tissue area divided by the cross-sectional area × 100. The asterisks indicate a statistically significant difference from IL-$6^{+/+}$ N-OVX ($P < 0.05$).

detected (Figure 5, and panels C and D of Figure 4). These findings demonstrate that IL-6 plays a key role in mediating the effects of estrogen withdrawal in bone, and that its absence appears to have a protective effect on trabecular bone.

The number of osteoclast precursors is not increased by ovariectomy in the IL-$6^{-/-}$ mice

The bone-resorbing osteoclasts originate in the bone marrow from hematopoietic precursors of the granulocyte–macrophage lineage. It has been shown that, as a consequence of

estrogen deficiency in mice, the number of colony forming units for this lineage (CFU-GM) is significantly increased, and this change correlates well with a corresponding increase of the osteoclast forming potential in the bone marrow of the same mice. These changes in the hematopoietic population are interpreted as the expression of one of the mechanisms by which bone resorption can be stimulated, that is the amplification of the osteoclastogenic potential (Jilka *et al.*, 1992). For this reason, a second set of ovariectomies was performed on a group of 2 month old mice, and the CFU-GM number was assessed by means of a colony forming assay with bone marrow cells from intact and ovariectomized IL-6$^{-/-}$ and $^{+/+}$ mice. As shown in Figure 7, the number of CFU-GM per tibia increased 3-fold in the ovariectomized wild type animals. However, the CFU-GM number did not change at all in the mutant mice, reproducing at the precursor level the lack of changes in the mature osteoclast population described in the previous section. As for the parameters of bone turnover, the basal CFU-GM level was somewhat higher in the IL-6$^{-/-}$ mice than in the wild type controls, although the difference was not found to be statistically significant.

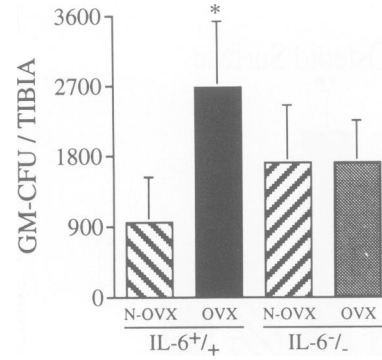

Fig. 7. Granulocyte–macrophage colony forming units assessed from the bone marrow of ovariectomized (OVX) and non-ovariectomized (N-OVX) IL-6$^{+/+}$ and IL-6$^{-/-}$ mice. Bars represent means ± SEM. The asterisk indicates a statistically significant difference from IL-6$^{+/+}$ N-OVX ($P < 0.05$).

Discussion

Because of its pronounced effects both *in vitro* and *in vivo*, IL-6 could be expected to play a crucial role in the development of the immune system and in the proliferation and differentiation of hematopoietic cells. However, we show here that IL-6 deficient mice are perfectly viable. Cytofluorimetric analysis on cell suspensions from spleen, mesenteric lymph nodes and thymus did not show any significant difference in the distribution of T cell subpopulations or in the percentage of activated T cells (V.Poli, S.Nuti and S.Abrignani, unpublished results). Moreover, the mutant mice did not show enhanced mortality following exposure to mouse hepatitis virus when raised in non-pathogen-free conditions (V.Poli, unpublished observation). While it is likely that detailed investigation will reveal specific defects in the immune response of the IL-6 deficient mice, it is conceivable that fundamental functions such as the differentiation of B and T lymphocytes cannot rely on a single factor, a manifestation of the redundancy in nature. In the case of IL-6, a possible mechanism for such redundancy can be identified in the fact that its effector molecule, gp130, is shared by the structurally related cytokines LIF, OncM, CNTF and IL-11 (reviewed by Taga and Kishimoto, 1992). The absence of IL-6 or of another family member might therefore be functionally compensated for by the action of the others, provided that their specific receptors are present. Interestingly, LIF deficient mice were also found to be viable. However, they have a specific defect in embryo implantation which leads to female sterility (Stewart *et al.*, 1992). This is an example in which functional specificity prevails over redundancy, presumably due to the absence in the uterine compartment of the other members of this cytokine subgroup or of their receptors. The uniqueness of the LIF function in implantation is consistent with our finding that IL-6 deficient females are fertile. As in the case of LIF for implantation, we could show that also IL-6 is not dispensable in some compartments, and that in particular it plays a unique role in the regulation of bone metabolism.

As mentioned above, bone metabolism is thought to be locally regulated by a network of cytokines produced by osteoblasts, bone marrow stromal cells and peripheral blood monocytes. A spontaneous mutation which inactivates the gene coding for one of these cytokines, the macrophage-colony stimulating factor (M-CSF) results in impaired bone resorption and leads to abnormal bone structure [the op/op mouse strain (Jedrzejczac *et al.*, 1990; Yoshida *et al.*, 1990)]. It has been shown that the specific defect of the op/op mice is due to an impaired ability of the CFU-GM in the bone marrow to differentiate, and as a consequence these mice are severely defective in macrophages and osteoclasts, both of which derive from this common precursor. Our results show that IL-6 is not essential for promoting osteoclastogenesis and osteoclastic bone-resorbing activity in mice with intact ovarian function, since non-ovariectomized IL-6 deficient mice have a normal number of osteoclasts and normally active bone resorption, as measured by the extent of eroded bone surface containing osteoclasts. In addition, also the number of osteoclast precursors in the bone marrow (CFU-GM) is normal. Accordingly, IL-6 is apparently not required for normal trabecular bone development, since neither gross structural abnormalities nor significant differences in trabecular bone volume were observed in the bones of the mutant mice (Figures 5C and D and 6A). In contrast, IL-6 appears to be important for cortical bone development, since a significantly lower percentage of cortical bone was present in the IL-6 deficient mice (Figure 6B). An explanation of the mechanism responsible for this abnormality, as well as of the apparently different roles played by IL-6 in trabecular and cortical bone metabolism, will require additional work.

The absence of IL-6 becomes crucial when the mice are subjected to estrogen depletion. Experimentally induced increased bone resorption leading to bone loss is in general coupled with increased bone formation, probably as an attempt of the organism to maintain the bone balance (reviewed by Rodgers and Monier-Faugere, 1993). From the results shown in Figures 4–7, it is clear that none of the changes induced by ovariectomy in the wild type mice occur in the IL-6 deficient mice. Neither bone resorption nor any parameters of bone formation are increased. As a consequence, the mice do not lose bone following ovariectomy, while the wild type littermates lose ~50% of their trabecular bone. Clearly, in mice IL-6 is required to generate the changes in bone metabolism which lead to higher

turnover and to bone loss. The additional finding that also the number of osteoclast precursors in the mutant mice is not changed following ovariectomy suggests that IL-6 function is already required at the stage of precursor recruitment. It could be argued that the lack of response to ovariectomy in the IL-$6^{-/-}$ mice could be due to an intrinsic defect in osteoclast function. However, the presence of a normal osteoclast number and osteoclast surface in the bones of the mutant mice, as well as the observation that the trabecular bone volume and the bone marrow cavities are normal, strongly indicate that basal osteoclast function is not deficient in the mutant mice. In contrast, what is altered is the capacity to enhance osteoclast number and function in response to estrogen depletion.

At this stage, we do not know the cause of the higher bone turnover in the IL-6 deficient mice. Since there are no experimental data suggesting that IL-6 may suppress basal bone remodeling, it seems reasonable to hypothesize that these effects are indirect, possibly due to deregulation of the levels of other cytokines and/or cytokine receptors.

IL-1 and TNFα are known to be potent stimulators of bone resorption. Like IL-6, they had been proposed to play an important role in induction of osteoclastogenesis and bone loss following estrogen depletion. It was shown that secretion of both IL-1 and TNFα by peripheral blood monocytes is increased in post-menopausal women and in both men and women with osteoporosis (Pacifici *et al.*, 1991). Interestingly, both cytokines can strongly induce IL-6 production in a variety of cell types. Particularly in osteoblast cell lines, this induction is inhibited by 17β-estradiol (Girasole *et al.*, 1992). Our results show that estrogen depletion does not increase bone resorption in the absence of IL-6, suggesting that both IL-1 and TNFα may act upstream in the cytokine cascade, exerting their function on bone at least partly through IL-6.

In conclusion, this study identifies IL-6 as a key molecule in determining the imbalance between bone resorption and bone formation caused by estrogen depletion in mice, and indicates it as a potential target for therapy of post-menopausal osteoporosis in women. In addition, analysis of bone cell function in IL-6 deficient mice will allow a better understanding of the mechanisms which regulate bone metabolism, under both physiological and pathological conditions.

Materials and methods

Vector construction

An IL-6 genomic clone (provided by T.Kishimoto) derived from a Balb/c genomic library (Tanabe *et al.* 1988), was used as a source of IL-6 genomic sequences. The 5′ homology, a 1 kb *SpeI*−*AatII* fragment corresponding to part of the 5′ non-coding region of the gene, was cloned blunt-ended in the *SmaI* site upstream of an MC1-Neo poly(A)$^+$ cassette from Stratagene (Thomas and Capecchi, 1987) previously cloned in the *Bam*HI site of a Bluescript BS$^+$ vector. An MC1 herpes simplex thymidine kinase (TK) cassette (Pevny *et al.* 1991) was inserted upstream of the 5′ homology. The direction of transcription of both the *Neo* and TK genes is indicated. The 3′ homology, an 8 kb *KpnI* fragment containing only the fourth and fifth exons, was cloned blunt-ended downstream from the *Neo* cassette. In the resulting construct, the *Neo* cassette replaces a 2.1 kb region containing the first three exons of the gene.

ES cell transfection and screening for recombinants

The construct was linearized by *NotI* digestion and used to electroporate 2×10^7 ES cells [from the ES cell line CCE, provided by E.Robertson (Robertson *et al.*, 1986)] with a Bio-Rad Gene Pulser. The cells were then plated on mitomycin C treated G418-resistant STO feeder cells, as described by Robertson (1987). G418 selection (Geneticin from Gibco, final effective concentration 200 μg/ml) was started 2 days after plating, and Gancyclovir selection (2×10^{-6} M) 5 days after plating. The use of Gancyclovir allowed an enrichment of ~5-fold. 240 doubly resistant clones were individually picked and plated into 24-well microtiter plates. Pools of five clones were analyzed by PCR, using a 5′ primer (5′P) located 50 bp upstream of the 5′ homology, and a 3′ primer (3′P) in the coding region of the *Neo* gene; this set of primers amplified a 1.6 kb fragment only in the homologous recombinants. A total of six clones that had undergone homologous recombination were identified, and analyzed by Southern blotting. Briefly, genomic DNA was digested with the indicated restriction enzymes, fractionated on a 0.8% agarose gel, transferred to a Nylon membrane (Hybond N$^+$) and hybridized to a radioactive probe. The probe used to discriminate between wild type and mutant allele (probe 5′, shown in Figure 1C) was a *Sau*3A−*Bsa*I 500 bp fragment which is included in the 5′ homology. A probe corresponding to the *Neo* gene was used to verify that only one copy of the vector had inserted in the genome. 5′ primer: TATGTACAGAGCCTACTTTCAAGCCTGG; 3′ primer: CCTGCGCTGACAGCCGGAACACGG.

Generation of IL-6 deficient mice

ES cell clones carrying the IL-6 mutation were injected into blastocysts of C57BL6 mice and transplanted into the uteri of F_1 (CBA × C57BL6) foster mothers. Male chimeras were mated to MF1 females, and agouti offspring (representing germline transmission of the ES genome) were screened for the presence of the targeted IL-6 locus by Southern blot analysis of *Eco*RI-digested tail DNA using the probe 5′. Female offspring heterozygous for the mutation were bred once with mice of the 129/SV/EV strain (Robertson *et al.*, 1987), the strain from which the CCE ES cells were derived. The resulting heterozygous offspring were bred together to generate mice homozygous for the mutation.

Northern blotting analysis, ELISA assay and 7TD1 bioassay

Total RNA was isolated from the spleen by the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987), fractionated by electrophoresis (20 μg per lane) and transferred to a GeneScreen membrane (NEN). The hybridization was carried out as described by Morrone *et al.* (1988) using as a probe a fragment containing the murine IL-6 cDNA sequence. After the hybridization, the probe was removed by heating for 1 min at 95°C, and rehybridized with a mouse β-actin probe as an internal control.

For the ELISA assay, a 96-well Falcon microtiter plate was coated with the rat anti-mouse IL-6 monoclonal MP5-20F3 from Pharmingen at a concentration of 1 μg/ml in 0.1 M $NaHCO_3$ pH 8.2. The plate was blocked for 6 h at room temperature with 3% BSA in PBS and incubated overnight with the samples at 4°C. Bound mIL-6 was revealed using a rabbit anti-mIL-6 polyclonal antibody and then a goat anti-rabbit IgG conjugated with alkaline phosphatase (Calbiochem 401326). The sensitivity of the assay corresponded to 0.06 ng of recombinant mIL-6.

The 7TD1 proliferation assay was performed as described (Van Snick *et al.*, 1986), in the absence or in the presence of 500 ng of the mAb MP5-20F3. The sensitivity of the assay corresponded to 0.5 pg of recombinant mIL-6.

Bone histomorphometry

Four-month old females were subjected to ovariectomy under ketamine anesthesia. All the animals were injected intraperitoneally with oxytetracycline (30 mg/kg body weight) on day 22 after surgery and with calcein (20 mg/kg body weight) on day 28 after surgery. On day 32 mice were sacrificed; one femur and one tibia per animal were fixed in 10% buffered formalin and processed undecalcified as described by Baron *et al.* (1983) in methylmethacrylate using a Hypercenter XP2 automatic tissue processor (Shandon). Longitudinal sections were obtained using a Polycut S microtome (Reichert-Jung). For each sample four sections were analyzed. Two sections (5 μm thick) were stained with Masson's trichrome and analyzed with bright field illumination, and two adjacent sections (10 μm thick) were analyzed unstained with UV light to identify fluorochrome labels. Fluorochrome labels are taken up by bone surfaces that are actively mineralizing. A double band of fluorescence establishes unequivocally that bone formation occurred during the time between administration of the two labels (Parfitt *et al.*, 1987).

Histomorphometric analysis was carried out in cancellous bone of the femoral metaphysis excluding cortical bone on 5 μm thick sections, and for the cortical bone on cross sections (70 μm thick) obtained at the level of the tibia−fibular junction, using the Bone Morphometry software (Bioquant System IV; R&M Biometrics, Nashville, TN), a computer with a digitizing board and a Nikon Labophot microscope equipped with both

visible and UV light sources and a camera lucida attachment. Histomorphometric parameters are reported according to the recommended ASBMR nomenclature (Parfitt *et al.*, 1987). The number of animals studied was as follows: IL-$6^{+/+}$ N-OVX: $n = 5$; IL-$6^{+/+}$ OVX: $n = 5$; IL-$6^{-/-}$ N-OVX: $n = 6$; IL-$6^{-/-}$ OVX: $n = 7$.

GM-CFU assay

Eight- to ten-week old mice were ovariectomized or left intact as controls. Three weeks after ovariectomy mice were sacrificed and bone marrow cells were flushed from both tibias. Nucleated cells were counted and 2.5×10^5 cells were plated in duplicate in α-MEM medium (Gibco) containing 0.5% agar and 0.1 mg/ml of kanamicin sulfate, supplemented with 10% heat inactivated fetal calf serum (Gibco), 100 U/ml of recombinant M-CSF (provided by D.Metcalf) and 40 ng/ml of recombinant mouse GM-CSF (Genzyme). Cultures were incubated for 6 days at 37°C in a humidified incubator under 5% CO_2 partial pressure, and the total number of colonies was counted.

Statistical analysis

All data are reported as mean ± SEM. Statistical differences were assessed using the Mann–Whitney U test (Hollander and Wolfe, 1973).

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