Transgenic Mice Expressing Soluble Tumor Necrosis Factor-Receptor are Protected Against Bone Loss Caused by Estrogen Deficiency

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Abstract

To evaluate the role of tumor necrosis factor (TNFα) in bone loss resulting from estrogen deficiency, the effects of ovariectomy were explored in six-month-old transgenic mice expressing high blood levels of a soluble TNF receptor type I (sTNFR1)-FcIgG3 fusion protein, which neutralizes TNFα, and in their nontransgenic littermates used as controls. These transgenic mice were identical to control mice in bone mass (evaluated by bone mineral density and content) and strength. 12 weeks after ovariectomy, the decrease in bone mass and increase in osteocalcin (marker of bone turnover) found in control mice were not observed in transgenic mice, which were not different from sham-operated mice, transgenic or not. This observation suggests a critical role for TNFα in the pathogenesis of bone loss induced by estrogen deficiency, a common cause of morbidity in postmenopausal women. (J. Clin. Invest. 1997. 99:1699–1703.)

Key words: osteoporosis • pathophysiology • DXA • osteocalcin • biomechanics

Introduction

Estrogen deficiency increases the rate of bone remodeling and leads to an imbalance between bone resorption and formation, resulting into net bone loss and osteoporosis. However, the exact pathogenesis of postmenopausal osteoporosis, which represents one of the most severe causes of morbidity in aged women, is still not fully understood. Cytokines like interleukin-1 (IL-1), tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), interleukin-11 (IL-11), and macrophage colony stimulating factor (M-CSF) may be involved in bone resorption by facilitating the recruitment and maturation of osteoclast precursors. Indirect evidence suggests that TNFα and IL-1, which both stimulate bone resorption in vitro (1, 2) and in vivo (3), might be involved in the initial step leading to bone loss induced by estrogen deficiency (3–14). TNFα and IL-1 are produced in increased amounts in vitro by peripheral blood monocytes of ovariectomized patients (15–17) and by bone marrow cells of ovariectomized rats (18), an effect antagonized by estrogen replacement therapy. This suggests that estrogens modulate the production of these cytokines and that estrogen deficiency might result in their increase and thus in enhanced bone resorption.

Recently, a model of transgenic mice in which the activity of TNFα is permanently prevented by the constitutive presence of high levels of circulating soluble TNFα-receptor1 (sTNFR1) has been described (19). In these mice, a transgene coding for a human sTNFR1-IgG3Fc fusion protein has been placed under the control of an alpha 1-anti-trypsin (AAT) promoter, leading to synthesis in the liver and release in the plasma of high amounts of the fusion protein (19). These mice showed an apparent complete neutralization of the systemic TNF and were protected from lethal septic shock and cerebral malaria. In contrast, they were highly sensitive to Listeria monocytogenes and Leishmania major infections (19).

To evaluate the role of TNF in bone loss resulting from estrogen deficiency, the effects of ovariectomy were explored in TNF-R1 transgenic mice and in their nontransgenic littermates used as controls. 12 weeks after ovariectomy, the decrease in bone mass and increase in bone turnover found in control mice were not observed in transgenic mice. This observation suggests a critical role for TNFα in the pathogenesis of bone loss induced by estrogen deficiency.

Methods

Animal. Transgenic mice expressing a soluble TNF-R1 fusion protein were generated as previously described (19). In the present study, a transgenic line (progeny of a founder AAT-sTNFR1-IgG3Fc transgenic mouse on a Balb/c background) expressing serum levels of 50–100 µg/ml of the fusion protein was used; littermate mice negative for the transgene, which have otherwise an identical genetic background, were used as controls. Mice were kept in cages of five, at 25°C with a 12/12 h light/dark cycle; they had free access to a diet (Kliba Mühle, Kaiseraugst, Switzerland) containing 0.8% phosphorus and 1.1% calcium during the whole experiment. At the age of 6 mo, ovariectomy or sham-operation were performed by dorsal approach under anesthesia induced by the intraperitoneal injection of ketamine hydrochloride (100 mg/kg body weight). All experimental designs and procedures have received the approval of the Animal Ethics Committee of the University of Geneva Faculty of Medicine.

Bone mass measurements by dual energy x-ray absorptiometry (DXA). Techniques measuring bone mineral content (BMC) and bone mineral density (BMD) by dual energy x-ray absorptiometry (20, 21) with a Hologic QDR-1000 instrument (Waltham, MA), were adapted for application in the mouse. An ultra high resolution mode (line spacing 0.0254 cm and resolution 0.0127 cm) was used with a collimator of 0.09 cm diameter. During the measurements, the animals were anesthetized with ketamine hydrochloride and were lying on the
back with the posterior legs maintained in external rotation with a tape. The hip, knee, and ankle articulations were in 90° flexion. Bone mineral content (BMC), bone mineral density (BMD) and scanned area were recorded at the level of the right tibia. To take into account possible differences in the local proportion of trabecular and cortical bone, the total tibia was divided into three equal regions. The proximal tibia corresponded to the region containing the largest proportion of trabecular bone, the midshaft was essentially formed by cortical bone. To evaluate accuracy, BMC of 12 tibias was measured in situ. Then, bones were isolated and ashed at 800°C. To evaluate accuracy, BMC of 12 tibias was measured in situ. Then, bones were isolated and ashed at 800°C. Calcium content in the ashes was determined. Highly significant linear regression was found between in situ BMC measurements and chemically determined calcium content ($y = -6.09 + 0.72 x; r^2 = 0.928; P < 0.0001$).

The influence of soft tissue modification was also evaluated by measuring tibia BMC/BMD under different thicknesses of muscle. This did not significantly influence BMC/BMD values (data not shown). The in vivo reproducibility was evaluated by measuring the coefficient of variation (CV = 100× SD/mean) of three BMC measurements in six mice, each time with repositioning of the animal. CV was 1.4% for the total tibia, 1.9% for the proximal tibia and 2.5% for the midshaft tibia. The stability of the measurement was controlled by scanning a phantom every week. The proximal tibia was selected for determination of bone mass, since it appeared to be particularly sensitive to estrogen deficiency (see Table II), probably due to its high proportion of trabecular bone. Measures of BMC and BMD have the advantage of evaluating bone mass as an end point parameter, thus integrating various alterations in bone turnover.

**Bone mechanical properties measurement.** Bone mechanical properties were evaluated at the level of the midshaft tibia using a three-point bending test (21). Tibia were excised and kept frozen at −20°C. Before mechanical testing, tibia were slowly thawed and then maintained immersed in physiological solution. The tibia was then placed in material testing machine on two supports separated by a distance of 8 mm and load was applied on the middle of the shaft. The mechanical resistance to failure was tested using a servo-controlled electromechanical system (Instron Corp., High Wycombe, England). The actuator was displaced at a rate of 2 mm/min. Both displacement and load were recorded. Ultimate strength (maximal load) and stiffness (slope of the linear part of the curve, representing the elastic deformation) were calculated. The reproducibility was evaluated by measuring the coefficient of variation (CV) between pairs of right and left tibia. CV was 2.6±0.5% ($n = 3$ pairs). A close relation between bone mass of the midshaft tibia as evaluated by in vivo DXA and biomechanics was found ($y = 0.923 x + 10.23; r^2 = 0.438, P = 0.008$).

**Bone histomorphometry.** The proximal tibial metaphyses were fixed, dehydrated and embedded undecalcified in a methylmethacrylate-based solution, as previously described (22). For each bone specimen, eight 7 μM thick longitudinal frontal sections were obtained using a Leica Polycut E microtome (Leica, France) equipped with tungsten carbide 50° knives. Sections were stained with Goldner’s trichrome. The histomorphometric analysis was performed with a Leica Quantimet Q570 color image processor equipped with a Sony 930 camera coupled to a Leitz DM/RBE microscope. Measurements were done on 8 sections per specimen at a magnification of $×25$, at the level of the secondary spongiosa. This area is located under the longitudinal bone growth area, or primary spongiosa.

Standardized terms and abbreviations are used, according to the American Society for Bone and Mineral Research histomorphometry nomenclature (23): BV/TV (expressed in percent), trabecular bone volume / tissue volume, corresponding to the amount of trabecular bone within the cancellous space; Tb. Th. (expressed in μm), mean thickness of the trabeculae; Tb. N. (expressed / mm) = mean number of trabeculae; Tb. Sp. (expressed in μm) = trabecular separation, estimating the distance between two trabeculae.

The last three parameters reflect the spatial distribution of trabeculae and are derived from surface to volume ratio (BS/BV) measurements according to stereologic formulae proposed by Parfitt (24).

**Biology.** Plasma osteocalcin was determined by radioimmunoassay with reagents of Biomedical Technologies (Stoughton, MA). Plasma calcium and phosphate levels were measured by atomic spectrophotometry and by a colorimetric assay described by Chen (25), respectively.

**Statistics.** Significance of difference between the groups was evaluated with an analysis of variance (ANOVA). When appropriate, a two sided Student’s $t$ test was performed. Histomorphometric results were compared using a non-parametric Kruskal-Wallis test.

**Results**

*Expression of high levels of soluble TNFR-IgG3 fusion protein does not modify bone metabolism in intact mice.* At 6 mo of age, transgenic mice and their littermate controls were comparable in body weight, bone size, bone mass as evaluated by BMD/BMC measurements, bone strength as assessed by a three-point bending test, and bone turnover as evaluated by serum osteocalcin levels (Table I). Stability of the BMC values observed in transgenic and control mice over 3 mo of observation indicated the maturity of the skeleton and ruled out major influences of the transgene expression on skeletal development (Fig. 1, Table II).

*Expression of high levels of soluble TNFR-IgG3 fusion protein protects against ovariecctomy-induced bone loss.* Ovariectomy performed at 6 mo of age was followed in control mice

**Table I. Characteristics of Control and Transgenic Mice at 6 mo of Age**

<table>
<thead>
<tr>
<th></th>
<th>Mice</th>
<th>Control</th>
<th>Transgenic</th>
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<tbody>
<tr>
<td></td>
<td>Body weight (grams)</td>
<td>27.3±0.54 (7)</td>
<td>28.0±0.5 (6)</td>
</tr>
<tr>
<td>Plasma</td>
<td>sTNFR1-FcIgG3 (ng/ml)</td>
<td>&lt; 5 (7)</td>
<td>50000 (6)</td>
</tr>
<tr>
<td></td>
<td>Osteocalcin (ng/ml)</td>
<td>128.3±12.3 (6)</td>
<td>135.9±8.7 (6)</td>
</tr>
<tr>
<td></td>
<td>Calcium (mmol/l)</td>
<td>2.15±0.04 (6)</td>
<td>2.17±0.05 (6)</td>
</tr>
<tr>
<td></td>
<td>Phosphate (mmol/l)</td>
<td>2.51±0.11 (6)</td>
<td>2.58±0.07 (6)</td>
</tr>
<tr>
<td>Tibia AREA cm²</td>
<td>0.1816±0.0043 (7)</td>
<td>0.1819±0.0056 (6)</td>
<td></td>
</tr>
<tr>
<td>DXA measurements</td>
<td>BMC (mg)</td>
<td>20.5±0.6 (7)</td>
<td>20.4±0.8 (6)</td>
</tr>
<tr>
<td></td>
<td>BMD (mg/cm²)</td>
<td>113.7±1.1 (7)</td>
<td>111.2±1.5 (6)</td>
</tr>
<tr>
<td>Tibia Ultimate strength</td>
<td>14.84±0.76 (4)</td>
<td>14.97±0.69 (4)</td>
<td></td>
</tr>
<tr>
<td>Biomechanics</td>
<td>Stiffness N/mm</td>
<td>1.33±0.14 (4)</td>
<td>1.15±0.15 (4)</td>
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</tbody>
</table>

Values are means±SEM (n) and were obtained in intact control and transgenic mice.
with a time-dependent decrease in the bone mass of the tibia, already detectable after 4 wk (Fig. 1); after 12 wk, these mice displayed a significant decrease \((P < 0.01)\) of proximal tibia BMD compared with sham-operated control mice (Table II and Fig. 1). Decrease in BMD of total tibia was of lower magnitude, although still significant \((P < 0.05)\); this could be explained by the fact that BMD of cortical bone was not altered, as seen by examination of the midshaft tibia (Table I). In contrast, ovariectomized transgenic mice did not display any bone loss during this period of time (Table II and Fig. 1) compared with sham-operated transgenic mice \((P < 0.01)\).

The bone mass decrease in ovariectomized control mice was also demonstrated by histomorphometric analysis as evaluated at the level of secondary spongiosa of proximal tibia sections of specimens collected 14 wk after ovariectomy or sham-operation. A significant decrease in bone volume, trabeculae number, and an increase in trabeculae separation were detected (Table III). However, there was no significant difference between sections from control mice, sham-operated and ovariectomized transgenic mice. The slight lower trabecular bone volume in transgenic sham-operated than in control mice did not reach a level of statistical significance. Osteocalcin levels, which reflect bone turnover, were increased 12 weeks after ovariectomy in control mice, but not in transgenic mice, in which they were on the contrary slightly decreased; this variation was not significant (Fig. 2).

**Discussion**

The present study demonstrates that the expression of soluble TNF receptor, neutralizing TNFα, protects mice from bone loss caused by estrogen deficiency. These observations suggest that TNFα alone is required for the induction of permanent bone loss resulting from estrogen deficiency. This conclusion must be evaluated in the light of other experimental evidence indicating a role for IL-1 and IL-6 in this condition. Recent studies using mutant mice with IL-1β gene deletion suggest that IL-1β is not required for post ovariectomy bone loss (26), but they do not exclude a role for IL-1α. Furthermore, administration of IL-1 receptor antagonist caused an incomplete prevention of rapid bone loss early after ovariectomy, but full prevention was achieved during this period only by the simul-

![Figure 1. Proximal tibia BMD (g/cm²) measured in 6-mo-old sham-operated (closed symbols) and ovariectomized (open symbols) mice. A represents values obtained in control mice and B in transgenic mice. Values of duplicate measurements are means±SEM, \(n = 5–6\). Significance of difference between groups was evaluated with an analysis of variance (ANOVA); *\(P < 0.001\).](image)
Table III. Effects of Ovariectomy on Histomorphometrical Variables in Control or Transgenic Mice

<table>
<thead>
<tr>
<th>BV/TV (%)</th>
<th>Sham</th>
<th>Ovariectomized</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>3.32 ± 0.54</td>
<td>1.57 ± 0.18*</td>
</tr>
<tr>
<td>Transgenic</td>
<td>2.45 ± 0.83</td>
<td>2.02 ± 0.21</td>
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<table>
<thead>
<tr>
<th>TB. N/mm</th>
<th>Sham</th>
<th>Ovariectomized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.41 ± 0.13</td>
<td>0.60 ± 0.101</td>
</tr>
<tr>
<td>Transgenic</td>
<td>1.10 ± 0.25</td>
<td>0.76 ± 0.07</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Tb. Sp. (μM)</th>
<th>Sham</th>
<th>Ovariectomized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>703.0 ± 68.0</td>
<td>1837.4 ± 305.1*</td>
</tr>
<tr>
<td>Transgenic</td>
<td>1093.0 ± 323.3</td>
<td>1323.4 ± 111.2</td>
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<table>
<thead>
<tr>
<th>Tb. Th. (μm)</th>
<th>Sham</th>
<th>Ovariectomized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.50 ± 1.85</td>
<td>27.20 ± 1.71</td>
</tr>
<tr>
<td>Transgenic</td>
<td>23.50 ± 1.55</td>
<td>26.60 ± 1.21</td>
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</tbody>
</table>

Values are means ± SEM. BV/TV, TB. N. Tb. Sp. and Tb. Th. were evaluated 14 wk after ovariectomy at the level of the secondary spongiosa of the proximal tibia in sham (n = 4) and ovariectomized (n = 5) transgenic and control mice. *P < 0.01 and 1P < 0.001 between sham and ovariectomized mice as evaluated by a Kruskal-Wallis nonparametric test.

An additional “downstream” effect of TNFα facilitating osteoclasts maturation, directly or through M-CSF release, may thus also be involved in bone loss resulting from estrogen deficiency. Under these conditions, our transgenic mice should prove highly useful to further clarify the chain of events leading to increased osteoclastogenesis in estrogen deficiency-induced osteoporosis. While there is evidence that TNF, IL-1, and IL-6 are involved in the very complex pathway operating in bone remodeling, and show a considerable degree of interplay, it appears from the present work that at least TNFα is required in remodelling occurring after estrogen depletion.

In conclusion, transgenic mice unable to use TNFα because of the expression of large amounts of soluble TNFα-receptor in the circulation are comparable to their non transgenic littermates in the development, the amount and mechanical quality of bone. This indicates that TNFα does not play a major regulating role in normal bone growth and modeling, or that its action can be replaced by that of other cytokines. These transgenic mice, however, appear protected against loss of trabecular bone and increased in bone remodeling resulting from ovariectomy. This suggests that an increment in TNFα production resulting from estrogen deficiency plays a critical role in the induction of increased bone turnover.

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