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Tumor necrosis factor-alpha: Alternative role as an inhibitor of osteoclast formation in vitro ☆

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Abstract

TNFα is known to stimulate the development and activity of osteoclasts and of bone resorption. The cytokine was found to mediate bone loss in conjunction with inflammatory diseases such as rheumatoid arthritis or chronic aseptic inflammation induced by wear particles from implants and was suggested to be a prerequisite for the loss of bone mass under estrogen deficiency. In the present study, the regulation of osteoclastogenesis by TNFα was investigated in co-cultures of osteoblasts and bone marrow or spleen cells and in cultures of bone marrow and spleen cells grown with CSF-1 and RANKL. Low concentrations of TNFα (1 ng/ml) caused a >90% decrease in the number of osteoclasts in co-cultures, but did not affect the development of osteoclasts from bone marrow cells. In cultures with *p55TNFR*$^{-/-}$ osteoblasts and *wt* BMC, the inhibitory effect was abrogated and TNFα induced an increase in the number of osteoclasts in a dose-dependent manner. Osteoblasts were found to release the inhibitory factor(s) into the culture supernatant after simultaneous treatment with $1,25(OH)_2D_3$ and TNFα, this activity, but not its release, being resistant to treatment with anti-TNFα antibodies. Dexamethasone blocked the secretion of the TNFα-dependent inhibitor by osteoblasts, while stimulating the development of osteoclasts. The data suggest that the effects of TNFα on the differentiation of osteoclast lineage cells and on bone metabolism may be more complex than hitherto assumed and that these effects may play a role in vivo during therapies for inflammatory diseases.

Keywords: TNFα; Osteoclast; Resorption; Inflammation; Glucocorticoid

Introduction

Preservation of bone mass and microarchitecture is essential for the mechanical stability and integrity of the skeleton. Changes in the structure of bone, which are caused by an imbalance between formation and resorption, constitute the base for the deterioration of the biomechanical properties of bone tissue that is observed in various pathological conditions [53]. These alterations in bone metabolism are governed by the local microenvironment regulating the proliferation, differentiation, activity, and survival of bone cells. In diseases like postmenopausal osteoporosis [15] and rheumatoid arthritis (RA) [22], the composition of the microenvironment is altered in favor of recruitment and/or activation of osteoclasts.

Tumor necrosis factor-alpha (TNFα) was found to be a major regulator in bone pathophysiology by stimulating bone resorption and inhibiting bone formation [43]. Levels of TNFα were found to be increased after surgical or natural menopause, a period characterized by increased bone turnover and loss of bone mass [27,46]. Using animal models of estrogen deficiency, the accelerated loss of bone mass was suggested to depend on TNFα [6,10,50]. The factor exerts its catabolic activity on bone by stimulating resorption [8,35] while inhibiting development and activity of osteoblast lineage cells [19,20].

TNFα signals through two cell-surface receptors, p55TNFR and p75TNFR which are expressed in bone tissue [32], though

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not equally [9], and which both activate the NFκB and MAP-(mitogen-activated protein) kinase pathways upon ligand binding (for recent review see [25]). The two receptors are not equivalent, with only the p55TNFR containing a death domain and the majority of the inflammatory actions induced by soluble TNFα being mediated through this receptor [5,42].

The local microenvironment regulating bone resorption in inflammatory diseases is characterized by an abundance of TNFα, interleukin-1 (IL1), and receptor activator of NFκB ligand (RANKL) [23,29,33]. While RANKL is essential for the development of osteoclasts [34], TNFα was found to be a non-essential but highly efficient regulatory factor [36]. It is, however, not clear, whether TNFα, or other growth factors, can replace RANKL during the development of osteoclasts [17,30,31], or whether low, but permissive levels or RANKL are an obligate requirement for osteoclastogenesis [36].

Recent evidence suggests that TNFα is not exclusively a catabolic modulator of bone metabolism. In models for fracture healing, deficiency in TNFα signaling led to an impaired healing process, with persistence of the cartilaginous fracture callus due to a failure to induce an apoptotic cascade in hypertrophic chondrocytes and the recruitment of osteoclasts [12,18,37].

In the present study, we describe TNFα to act not only as a stimulator of bone resorption, but the cytokine may also exert inhibitory effects on the development of osteoclasts in vitro. Using cells from mice deficient in the p55TNFR, our results demonstrate that inhibition of osteoclastogenesis by TNFα is mediated via cells of the osteoblast lineage, while the stimulation is mediated at least in part through an effect on the hematopoietic precursor cells. The inhibitory effect of TNFα is counteracted by dexamethasone (Dex), suggesting that this effect might contribute to the imbalance in bone metabolism under glucocorticoid treatment.

Materials and methods

Animals

Mice deficient in the p55TNFR on C57Bl/6 background [51], C57Bl/6 wild-type (*wt*) and *ddy* [59] mice, were housed in the Central Animal Facility of the Medical Faculty of the University of Berne, complying with the Swiss and the US National Institutes of Health guidelines for care and use of experimental animals. The experiments in this study were approved by the State Committee for the Control of Animal Experimentation.

Cell cultures

To follow the development of osteoclasts in vitro, bone marrow cells (BMC) were either cultured with colony-stimulating factor-1 (CSF-1) and RANKL, or co-cultures were performed with osteoblasts and BMC [61] or spleen cells [59].

BMC were isolated from 6-week-old mice by flushing the bone marrow from the excised femora with cell culture medium [αMEM containing 10% FBS (Inotech AG, Dottikon, Switzerland) and pen/strep (100 U/ml and 100 μg/ml, respectively; GIBCO BRL Life Technologies, Basle, Switzerland)]. After centrifugation at 250 × *g* for 10 min at 4°C, the cell pellet was resuspended in medium. Spleen cells were isolated from 2-week-old mice by filtering the disaggregated tissue through a 100 μm mesh in MEM Hank's [62]. For lyses of the erythrocytes, the cells were resuspended in 0.727% NH_4Cl/0.17% Tris–HCl, pH 7.2 for 20 min on ice. Osteoblasts were isolated from calvariae of 1–2-day old mice by sequential collagenase digestion [65]. Subsequently, the cells were expanded for 4 days in culture medium, and thereafter stored in liquid nitrogen at a concentration of 10^6 cells/ml.

BMC were grown in 24-well plates (Falcon, Fisher Scientific AG, Wohlen, Switzerland) at a density of 7.5×10^5 cells/well in 0.5 ml cell culture medium, supplemented with 30 ng/ml CSF-1 (kindly provided by Cetus Corporation, CA) and 50 ng/ml human recombinant soluble RANKL (Juro GmbH, Lucerne, Switzerland). The medium was changed at day 3 of the culture and osteoclasts developed within 4–6 days. In co-cultures of primary osteoblasts and BMC, 10^4 osteoblasts and 1.5×10^5 BMC or 5×10^5 spleen cells were grown in 24-well plates in cell culture medium, supplemented with 10^{-8} M $1,25(OH)_2D_3$ (Hoffmann-La Roche, Basle, Switzerland). The medium was changed after 3 days and osteoclasts developed within 6 days.

To visualize osteoclasts, the cell cultures were stained for the marker enzyme tartrate resistant acid phosphatase (TRAP), using a commercially available kit (SIGMA Diagnostics, Buchs, Switzerland), and TRAP positive cells with 3 and more nuclei were counted as multinucleated osteoclast-like cells.

In the experiments, in which TNFα (Genentech, San Francisco, CA, USA) was added to the cultures, the growth factor was added at 1 ng/ml, unless stated otherwise, during the time periods indicated in Results.

Preparation and test of conditioned media (CM)

To investigate, whether osteoblasts upon treatment with TNFα release a soluble activity into the cell supernatant, 10^4 primary osteoblasts were seeded into 24-well plates in 0.5 ml of cell culture medium supplemented with 10^{-8} M $1,25(OH)_2D_3$ and TNFα (1 ng/ml). The CM was collected after 3 and 6 days, respectively. The effect of CM on the formation of TRAP-positive osteoclasts was tested in co-cultures and in cultures of BMC, replacing 10% of the growth medium with CM collected on day 3 during the first 3 days and with CM collected on day 6 during days 4–6 of the cultures, if not indicated otherwise.

Quantitative real-time PCR

To determine the levels of mRNAs encoding RANKL and OPG, and of known inhibitors of osteoclastogenesis synthesized by osteoblasts in response to treatment with TNFα at 1 ng/ml, the cells were seeded into 24 well plates (10^4 cells/well) and grown for 72 h. Total RNA was isolated using the RNeasy Mini Kit from Qiagen (Basle, CH), according to the recommendations of the manufacturer. The total RNA was reverse transcribed using MMuLV reverse transcriptase (Roche Diagnostics, Rotkreuz, CH) and random hexamers (Promega, Catalys AG, Wallisellen, CH). For Amplification in an ABI PRISM 7700 system (Applied Biosystems, Rotkreuz, CH), the following Assays-on-Demand (Applied Biosystems, Rotkreuz, CH) were used: RANKL (Mm00441908_m1), OPG (Mm00435452_m1), IL4 (Mm00445259_m1), IL10 (Mm00439616_m1), IL12a (Mm00434165_m1), IL13 (Mm00434206_m1), IFNβ (Mm00439546_s1), IFNγ (Mm00801778_m1), and CSF-1 (Mm00432688_m1). The C_T values were normalized against 18S rRNA (Hs99999901_s1). The reactions were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Rotkreuz, CH), Assay-on-Demand mixtures diluted 1:20, and 5 ng (0.05 ng for 18S rRNA) cDNA. The reaction mixes were preincubated for 2 min at 50°C, followed by 10 min at 95°C. Thereafter, 45 cycles of 15 s at 95°C and 1 min at 60°C each were performed. The reactions were analyzed using the ABI PRISM sequence detection application software SDS V1.2.

Determination of OPG

OPG protein levels were determined in supernatants of osteoblasts treated ± $1,25(OH)_2D_3$ (10^{-8} M) and TNFα (1 ng/ml), using a Quantikine® Immunoasay (R&D Systems GmbH, Wiesbaden, Nordenstadt, Germany) as recommended by the manufacturer.

Inhibition of growth factors with antibodies

Specific inactivating rat antibodies against murine TNFα (BD Pharmingen, Basle, Switzerland) were used to differentiate between the inhibitory effects of TNFα on in vitro osteoclastogenesis and the induction of the inhibitory

activity by TNFα. To block TNFα effects in the co-culture and in osteoblasts, the antibody was added to the cells, at a concentration of 0.1 μg/ml, simultaneously with TNFα during the whole culture period. To assess the contribution of the cytokine in CM, the media were preincubated for 30 min at room temperature with anti-TNFα antibodies (0.1 μg/ml) and thereafter added to the cell cultures. To inhibit GM-CSF, CM were preincubated for 30 min at room temperature with rat anti-mouse GM-CSF antibodies (BD Pharmingen, Basle, Switzerland).

Statistical analysis

Differences in the development of OCLs were evaluated by two-tailed, unpaired Student's *t* test (Excel; Microsoft, Redmond, WA, USA).

Results

TNFα exerts stimulatory and inhibitory effects on osteoclastogenesis

To assess the effects of TNFα on the development of osteoclasts, co-cultures of *ddy* osteoblasts and *ddy* BMC and cultures of *ddy* BMC (CSF-1: 30 ng/ml; RANKL 50 ng/ml) were grown in the presence of 1 ng/ml of the cytokine (Fig. 1A). In the co-cultures, TNFα inhibited the development of osteoclasts by more than 90%. In cultures of *ddy* BMC, however, TNFα did not affect the formation of osteoclasts. Since soluble TNFα is thought to act through the p55TNFR, different doses of the growth factor were added to co-cultures of C57Bl/6 *wt* or *p55TNFR*$^{-/-}$ osteoblasts and *ddy* BMC (Fig. 1B). While the development of osteoclasts was inhibited in C57Bl/6 *wt*/*ddy* co-cultures at concentrations of TNFα of 0.1 ng/ml and higher, the growth factor failed to exert an inhibitory effect in *p55TNFR*$^{-/-}$/*ddy* co-cultures. To the contrary, the development of osteoclasts was stimulated significantly at concentrations of TNFα of 1 ng/ml and above. It should be noted, however, that the efficiency of osteoblasts of *C57Bl/6* background to support the development of osteoclasts was reduced in comparison to osteoblasts of *ddy* origin.

The effects of TNFα are not mediated by stromal cells in the BMC

To exclude the possibility of stromal cells in the BMC being responsible for the effects of TNFα on osteoclastogenesis, co-cultures with *ddy* or *p55TNFR*$^{-/-}$ osteoblasts and *ddy* spleen cells were performed. The development of osteoclasts was inhibited in *ddy*/*ddy* cultures in dependence of the dose of TNFα, significant inhibition being observed at 1 ng/ml (Fig. 2A). In *p55TNFR*$^{-/-}$/*ddy* co-cultures (Fig. 2B), TNFα caused an increase in the number of osteoclasts at 1 ng/ml when the cells were grown with 1,25$(OH)_2D_3$ and at 5 ng/ml in cultures grown without 1,25$(OH)_2D_3$.

TNFα stimulates osteoclastogenesis in cultures of BMC and spleen cells

Previously, TNFα was described to stimulate the development of osteoclasts from hematopoietic precursor cells [36]. Since the data presented above suggest an inhibitory activity of TNFα, BMC (Figs. 3A and B) and spleen cells (Figs. 3C and D) were cultured with CSF-1 (3 ng/ml in BMC and 30 ng/ml in spleen cell cultures) and varying concentrations of RANKL (0, 0.5, 5, and 50 ng/ml) and TNFα (0–50 ng/ml). In BMC and spleen cells from *ddy* mice, TNFα stimulated the development of osteoclasts in vitro at 5 ng/ml and higher, if RANKL was either absent or present at low concentrations (0.5 ng/ml). If RANKL was added to the cultures at 5 and 50 ng/ml, respectively, TNFα was not able to stimulate osteoclastogenesis further (Figs. 3A and C). Addition of TNFα to cultures of BMC and spleen cells from *p55TNFR*$^{-/-}$ mice did not result in an increase in the development of osteoclasts (Figs. 3B and D). In

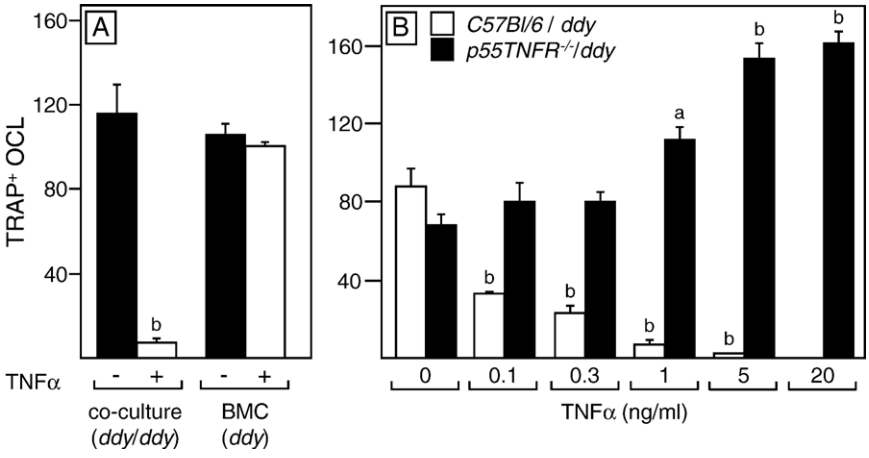

Fig. 1. TNFα exerts dual effects on osteoclastogenesis. TNFα (1 ng/ml) was added to co-cultures of *ddy*, C57Bl/6 *wt*, and *p55TNFR*$^{-/-}$ osteoblasts and *ddy* BMC and to cultures of *ddy* BMC supplemented with 30 ng/ml CSF-1 and 50 ng/ml and RANKL. (A) Addition of 1 ng/ml of TNFα to *ddy*/*ddy* co-cultures caused a significant decrease in the number of osteoclasts as compared to the cultures without TNFα, but remained without effect in cultures of *ddy* BMC. (B) In co-cultures of C57Bl/6 *wt* osteoblasts with *ddy* BMC, TNFα inhibited the development of osteoclasts in a dose-dependent manner. In *p55TNFR*$^{-/-}$/*ddy* co-cultures, the inhibitory effect of TNFα was abrogated, and increasing doses of TNFα stimulated the development of osteoclasts. Bars represent the average ± SEM of 5 wells from one representative experiment. (a) $P < 0.05$ and (b) $P < 0.01$; significant difference to controls without TNFα.

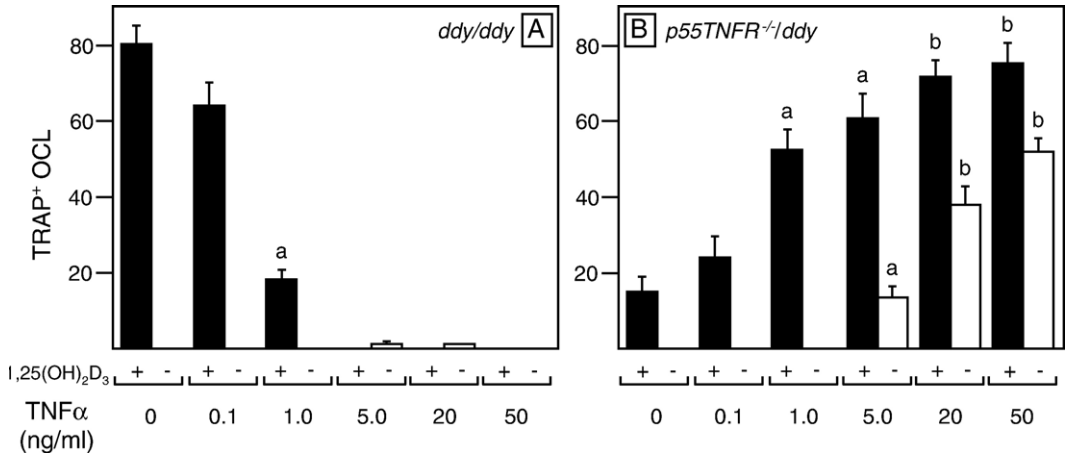

Fig. 2. TNFα inhibits osteoclastogenesis indirectly through an effect on osteoblasts precursors. The effects of TNFα on the development of osteoclasts were determined in co-cultures of *ddy* (A) and *p55TNFR*$^{-/-}$ (B) osteoblasts and *ddy* spleen cells in the presence or absence of $1,25(OH)_2D_3$. In *ddy*/*ddy* co-cultures with $1,25(OH)_2D_3$, TNFα exerted a dose-dependent inhibitory effect on osteoclastogenesis. A few osteoclasts developed with high concentrations of TNFα, in the absence of $1,25(OH)_2D_3$, but the numbers remained low. In *p55TNFR*$^{-/-}$/*ddy* co-cultures grown with $1,25(OH)_2D_3$, TNFα caused a significant increase in the number of osteoclasts at concentrations of 1 ng/ml and higher. In the absence of $1,25(OH)_2D_3$, no osteoclasts developed without TNFα, but at 5 ng/ml and higher, the factor stimulated osteoclastogenesis. Bars represent the average ± SEM of 5 wells from one representative experiment. (a) $P < 0.05$ and (b) $P < 0.01$; significant difference from controls without TNFα.

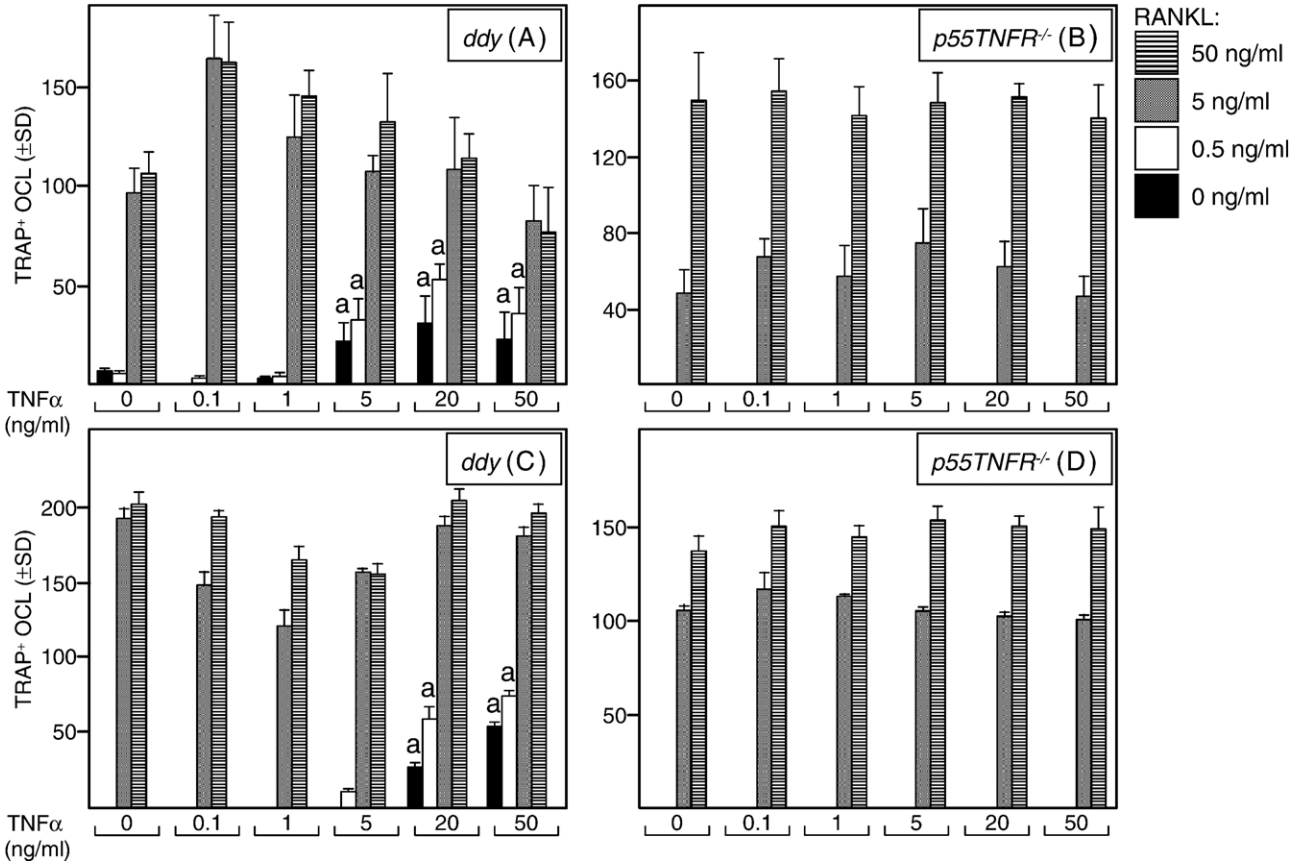

Fig. 3. TNFα stimulates the formation of osteoclasts from hematopoietic precursors. BMC (A, B) and spleen cells (C, D) from *ddy* (A, C) and p55TNFR$^{-/-}$ (B, D) mice were grown with different doses of RANKL and TNFα. When *ddy* BMC and spleen cells were grown with 0 or 0.5 ng/ml RANKL, TNFα dose dependently stimulated osteoclastogenesis. At RANKL concentrations of 5 and 50 ng/ml, however, the number of osteoclasts was not significantly affected by TNFα. In BMC and spleen cells from *p55TNFR*$^{-/-}$ mice, osteoclasts were formed in the presence of high levels of RANKL (5 and 50 ng/ml), and TNFα did not affect the number of osteoclasts at all concentrations of RANKL. Bars represent the average ± SEM of 6 wells from one representative experiment. (a) $P < 0.05$; significant difference from controls without TNFα.

the absence of RANKL, and with low levels of the growth factor (0.5 ng/ml), no osteoclasts were detected in the cultures, while at 5 ng/ml and 50 ng/ml, the number of osteoclasts was not changed by TNFα at any dose.

Osteoblasts release an inhibitor of osteoclastogenesis in response to TNFα

To assess, whether the inhibition of osteoclastogenesis by TNFα requires cell–cell contact, or whether it is mediated through a soluble factor, CM from C57Bl/6 *wt* or *p55TNFR*$^{-/-}$ osteoblasts (treated with TNFα at 1 ng/ml for 72 h) were added at a final concentration of 10% to *ddy*/*ddy* co-cultures (Fig. 4A). This concentration of CM was previously found to mediate a full effect, while the respective control media did not affect the culture (results not shown). CM from C57Bl/6 *wt* osteoblasts treated with 1,25$(OH)_2D_3$ and TNFα caused a decrease in the number of osteoclasts of more than 90%. When C57Bl/6 *wt* osteoblasts were treated with TNFα in the absence of 1,25$(OH)_2D_3$, osteoclastogenesis was reduced by the CM by ca. 25% due to carry over of residual TNFα. CM from *p55TNFR*$^{-/-}$ osteoblasts treated with TNFα caused a decrease of ca. 35% in the number of osteoclasts, independent of the presence of 1,25$(OH)_2D_3$ in the osteoblast culture, this inhibition again being caused by residual TNFα in the CM. In *p55TNFR*$^{-/-}$/*ddy* co-cultures, CM from C57Bl/6 *wt* osteoblasts treated with TNFα and 1,25$(OH)_2D_3$ inhibited osteoclastogenesis by ca. 80% (Fig. 4B). CM from *p55TNFR*$^{-/-}$ osteoblasts did not affect osteoclastogenesis, and the residual inhibition due to TNFα carried over with the CM into the co-culture was no longer detectable. Lastly, the development of osteoclasts in cultures of *ddy* BMC, grown with CSF-1 (30 ng/ml) and RANKL (50 ng/ml) was not affected by TNFα (1 ng/ml) added directly to the cells, but the CM from C57Bl/6 *wt* osteoblasts caused a reduction of the number of osteoclasts by more than 95% (Fig. 4C).

Anti-TNFα antibodies block the effect of TNFα, but not the one of the CM

To differentiate between the effects of TNFα and of the TNFα-dependent inhibitory activity, *ddy*/*ddy* co-cultures were grown in the presence or absence of TNFα and anti-TNFα antibodies (Fig. 5A). TNFα alone blocked osteoclastogenesis in dependence of the dose added to the co-cultures (1 ng/ml and 5 ng/ml). The development of osteoclasts was restored by the addition of anti-TNFα antibodies, while control rat IgG antibodies added at the same concentration (0.1 μg/ml) remained without effect. Addition of anti-TNFα antibodies to *ddy* osteoblasts simultaneously with TNFα and 1,25$(OH)_2D_3$ during the conditioning phase blocked the release of the inhibitory activity, rendered the CM ineffective, and thus allowed for the development of osteoclasts (Fig. 5B). Pretreatment of CM from *ddy* osteoblasts, treated with 1,25$(OH)_2D_3$ and TNFα, with anti-TNFα antibodies, however, did not reverse the inhibitory effect on the development of osteoclasts in *ddy*/*ddy* co-cultures (Fig. 5C).

The inhibitory activity accumulates in CM and inhibits proliferation/differentiation of early precursors

To investigate the release of the TNFα-dependent inhibitor of osteoclastogenesis over time, CM from *ddy* osteoblasts grown with TNFα and 1,25$(OH)_2D_3$ were collected after 24 h, 48 h, and 72 h (Fig. 6B) and added to *ddy*/*ddy* co-cultures at a final concentration of 10%. The CM collected after 24 h inhibited the development of osteoclasts by approximately 60%. CM collected after 72 h

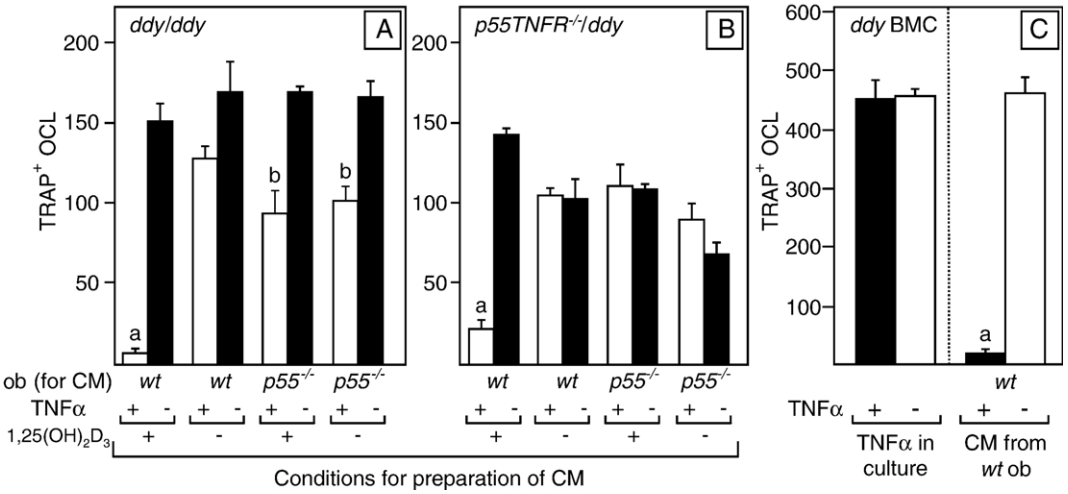

Fig. 4. TNFα induces osteoblasts to release a soluble inhibitor of osteoclastogenesis. CM were collected from C57Bl/6 *wt* and *p55TNFR*$^{-/-}$ osteoblasts grown in the presence or absence of TNFα (1 ng/ml) and 1,25$(OH)_2D_3$ (10^{-8} M) for 72 h. The effects of the CM on osteoclastogenesis were tested in *ddy*/*ddy* (A) and in *p55TNFR*$^{-/-}$/*ddy* (B) co-cultures and in cultures of *ddy* BMC (C). Addition of CM from C57Bl/6 *wt* osteoblasts treated with TNFα/1,25$(OH)_2D_3$ induced a significant inhibition in the number of osteoclasts in both *ddy*/*ddy* and *p55TNFR*$^{-/-}$/*ddy* co-cultures. The effects of CM from *p55TNFR*$^{-/-}$ osteoblasts were independent of a simultaneous treatment of the cells with 1,25$(OH)_2D_3$. When CM from C57Bl/6 *wt* osteoblasts was added to cultures of *ddy* BMC, the development of osteoclasts was inhibited, while direct addition of TNFα did not reduce the number of osteoclasts. Bars represent the average ± SEM of 3 wells from one representative experiment. (a) $P < 0.01$ [C57Bl/6 *wt* CM] and (b) $P < 0.05$ [*p55TNFR*$^{-/-}$ CM]; significant difference from controls without TNFα.

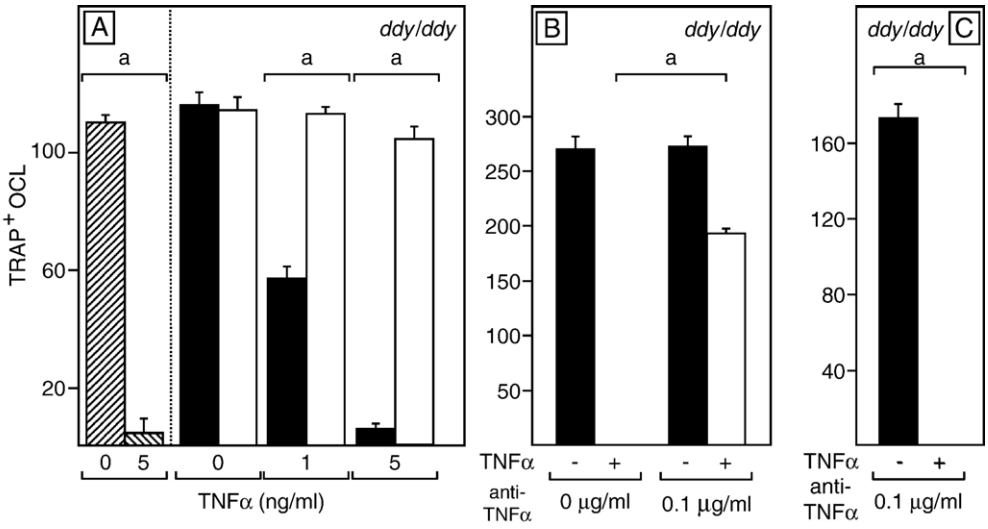

Fig. 5. Anti-TNFα antibodies block the release of the inhibitory activity by osteoblasts. To differentiate between the effects of the osteoblast-derived inhibitor of osteoclastogenesis and of TNFα, anti-TNFα antibodies and control rat IgG were added simultaneously with TNFα to *ddy*/*ddy* co-cultures (A). Addition of anti-TNFα antibodies (white bars) blocked the effect of TNFα in the co-cultures and the development of osteoclasts was restored (black bars: cultures without anti-TNFα antibodies). The unspecific control antibodies did not affect the co-culture (hatched bars: 0.1 µg/ml rat IgG ± TNFα). Addition of anti-TNFα antibodies simultaneously with TNFα to *ddy* osteoblasts during the preparation of CM decreased the release of the inhibitory activity into the CM and osteoclastogenesis was restored (B). Pretreatment of the CM from *ddy* osteoblasts treated with TNFα and $1,25(OH)_2D_3$ with anti-TNFα antibodies, however, did not restore the development of osteoclasts, demonstrating that the inhibitory activity in the CM is not identical to TNFα (C). Bars represent the average ± SEM of 5 wells from one representative experiment. (a) $P < 0.01$; significant difference from control without TNFα.

caused a decrease in the number of osteoclasts of 90% and more. In control co-cultures, to which 1 ng/ml of TNFα was added directly, osteoclastogenesis was inhibited by more than 90% (Fig. 6A). In order to determine whether the inhibitory activity released by osteoblasts in response to the treatment with TNFα acts during the early or late stages of osteoclastogenesis, the CM were added to *ddy*/*ddy* co-cultures during the complete culture period (days 0–6), during days 0–3 (early), and during days 4–6 (late). When the CM were added to the co-cultures either between days 0–3 and day 0–6, the development of osteoclasts was reduced by more than 95% (Fig. 6C). Addition of CM to the co-cultures between days 4 and 6, however, caused only a slight, not significant, reduction in osteoclast numbers.

TNFα inhibits osteoclastogenesis not through the OPG/RANKL system or GM-CSF

The OPG/RANKL system is known to be a potent regulator of osteoclastogenesis and therefore the expression of OPG and

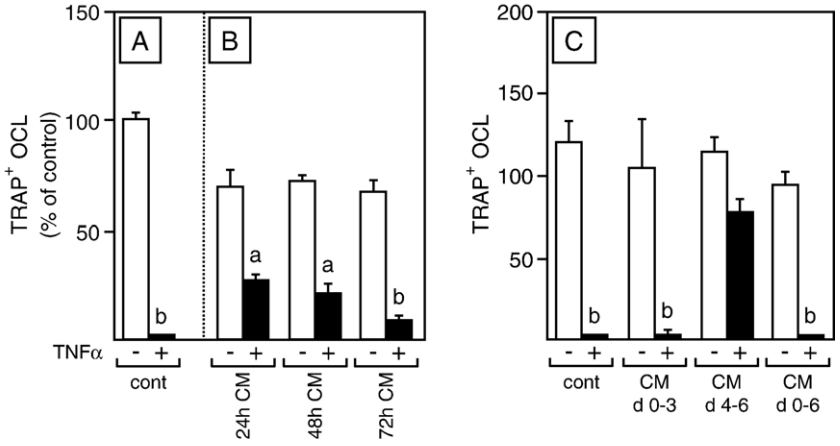

Fig. 6. The inhibitory activity accumulates in the CM and inhibits early stages of osteoclast development. CM from *ddy* osteoblasts, treated or not treated with TNFα (1 ng/ml), were collected after 24 h, 48 h, and 72 h and added to *ddy*/*ddy* co-cultures (B). The CM became more potent in inhibiting osteoclastogenesis with extended conditioning periods. Control *ddy*/*ddy* cultures, to which TNFα was added, showed inhibition of >90% (A). To assess, whether the inhibitory activity affects primarily early, proliferating osteoclast precursors or late, differentiating cells, the CM were added during the complete culture period (days 0–6), or during the early (days 0–3) or late (days 4–6) phase only. In the control, TNFα was added to the co-culture (C). Addition of the CM during days 0–6 and days 0–3 blocked the development of osteoclasts nearly completely, while addition of the CM during days 4–6 allowed for almost undisturbed osteoclastogenesis. Bars represent the average ± SEM of 3 wells from one representative experiment. (a) $P < 0.05$ and (b) $P < 0.01$; significant difference from controls without TNFα.

RANKL in response to TNFα was investigated at mRNA and protein levels. The levels of transcripts encoding OPG were greatly suppressed by $1,25(OH)_2D_3$ (10^{-8} M) after 3 days in *ddy* osteoblasts. Addition of TNFα caused a decrease of OPG mRNA in the absence of $1,25(OH)_2D_3$, but the already low levels of OPG mRNA in the presence of the hormone were not further affected by the cytokine (Fig. 7A). Exposing *ddy* osteoblasts to $1,25(OH)_2D_3$ led to a significant increase in the levels of transcripts encoding RANKL (Fig. 7B). While TNFα caused a slight, though non-significant, increase in the levels of RANKL mRNA in the absence of $1,25(OH)_2D_3$, the factor did not affect transcript levels in the presence of the hormone. Protein levels of OPG in the CM after treatment with $1,25(OH)_2D_3$ behaved similar to transcript levels. OPG concentrations were reduced by more than 95%, when $1,25(OH)_2D_3$ was added to the cultures (Fig. 7C). TNFα caused a slight decrease of OPG levels in the absence of $1,25(OH)_2D_3$, but did not further affect the already low levels of OPG in the presence of the hormone. Since GM-CSF was previously found to be regulated by TNFα and to be a potent inhibitor of osteoclastogenesis, its putative role in the TNFα induced inhibition of osteoclastogenesis was assessed by using inactivating antibodies. For this purpose, CM from osteoblasts grown with TNFα and $1,25(OH_2D_3$ for 3 days were pretreated with rat anti-mouse GM-CSF antibodies and subsequently added to *ddy*/*ddy* co-cultures at a concentration of 10% (Fig. 7D). While a slight recovery of osteoclastogenesis to about 15% of control values was observed in the presence of low concentrations of the antibody, the number of osteoclasts was not restored above this threshold. The expression of further putative inhibitors of osteoclastogenesis was determined by quantitative RT-PCR. No evidence was found that levels of RNAs encoding CSF-1 [16], IL4 [55], IL10 [66], IL12α [28], IL13 [45], IFNβ [13], and IFNγ [60] were increased in primary osteoblasts treated with TNFα and $1,25(OH)_2D_3$ (data not shown).

Dexamethasone blocks the release of the inhibitory activity induced by TNFα

Dex has previously been shown to support osteoclastogenesis and to stimulate differentiation of osteoblast lineage cells. The combined effects of Dex, $1,25(OH)_2D_3$, and TNFα on the release of the TNFα-dependent inhibitor of osteoclastogenesis by *ddy* (Fig. 8A) and *p55TNFR*$^{-/-}$ (Fig. 8B) osteoblasts were investigated by testing the CM in *ddy*/*ddy* co-cultures. In the absence of TNFα, CM from *ddy* and *p55TNFR*$^{-/-}$ osteoblasts did not affect osteoclastogenesis, and addition of Dex during the conditioning phase induced an increase in the number of osteoclasts. CM from *ddy* osteoblasts treated with $1,25(OH)_2D_3$ and TNFα inhibited the development of osteoclasts in co-cultures, in contrast to CM from *p55TNFR*$^{-/-}$ osteoblasts. However, CM from *ddy* osteoblasts grown with $1,25(OH)_2D_3$, TNFα, and Dex, did not inhibit the development of osteoclasts in co-cultures any longer. To the contrary, not only was the release of the inhibitory activity blocked, but also the stimulatory effect of Dex was maintained. Furthermore, addition of Dex to the culture media during the conditioning phase blunted the

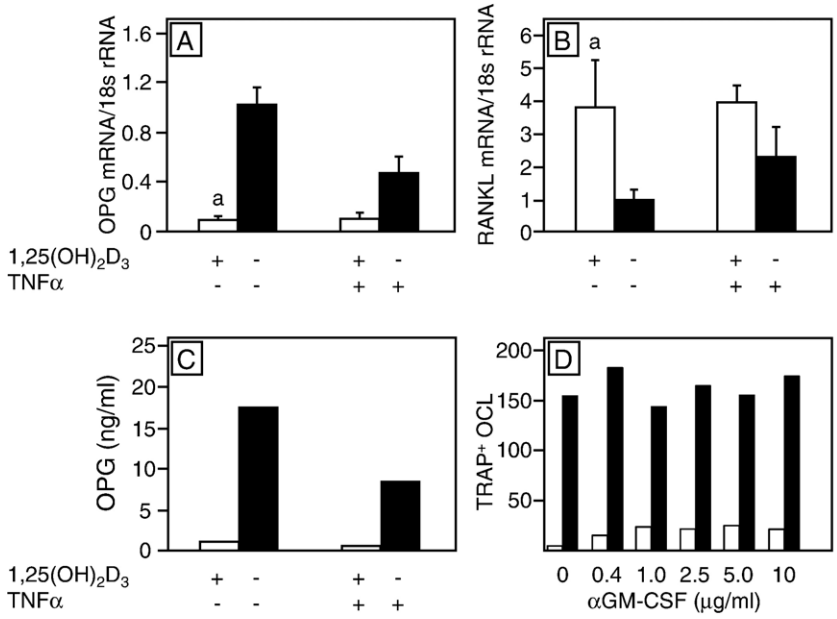

Fig. 7. The inhibitory activity is not OPG or GM-CSF. Levels of transcripts encoding OPG (A) and RANKL (B) were determined in *ddy* osteoblasts after growth for 72 h with and without $1,25(OH)_2D_3$ and TNFα. OPG mRNA levels were strongly repressed by $1,25(OH)_2D_3$. TNFα slightly decreased OPG mRNA in the absence of $1,25(OH)_2D_3$, but did not further affect the low levels of transcripts in the presence of the hormone. Levels of mRNA encoding RANKL were stimulated by $1,25(OH)_2D_3$, and again, TNFα did not exert a further significant effect. OPG protein levels were decreased by more than 95% in the presence of $1,25(OH)_2D_3$ and these levels were not affected by TNFα (C). The activity of GM-CSF, another potent inhibitor of osteoclastogenesis, in CM was blocked with rat anti-mouse GM-CSF antibodies (D). In the presence of the antibody, the development of osteoclasts was slightly increased, but never exceeded 15% of the control values.

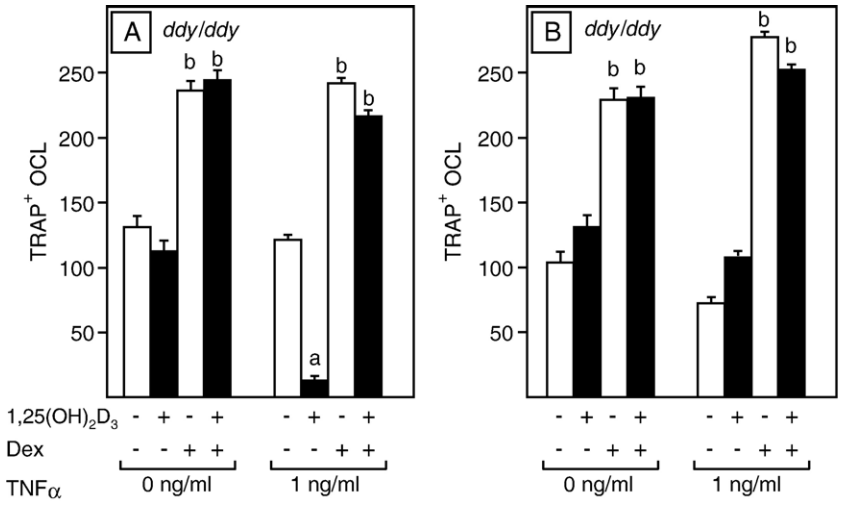

Fig. 8. Dexamethasone blocks the release of the inhibitor of osteoclastogenesis. CM from *ddy* (A) and *p55TNR*$^{-/-}$ (B) osteoblasts were produced with or without 1,25(OH)$_2$D$_3$, TNFα, and Dex (10^{-8} M), and tested on co-cultures of *ddy* osteoblasts and *ddy* BMC. Dex alone caused an increase in the number of osteoclasts. When added together with TNFα to the osteoblast cultures, Dex blocked the release of the TNFα-dependent inhibitory activity, while maintaining its stimulation of osteoclastogenesis. Bars represent the average ± SEM of 5 wells from one representative experiment. (a) $P < 0.01$; significantly different from controls without TNFα; and (b) $P < 0.01$; significant difference from controls without Dex.

differences of the effects of the CM from *ddy* and *p55TNFR*$^{-/-}$ osteoblasts, respectively, on the development of osteoclasts.

Discussion

In this study, we present data suggesting that TNFα, besides its well characterized action as a stimulator of osteoclastogenesis and bone resorption in vitro and in vivo, under specific conditions may exert an inhibitory activity on the development of cells of the osteoclast lineage.

In the past, the role of TNFα and of the TNF receptors (p55TNFR and p75TNFR) in the development of osteoclasts has been investigated in detail. Both receptors bind the homotrimeric ligand [11], but it is the p55TNFR which mediates primarily the effects of soluble TNFα, while p75TNFR is activated by the membrane-bound factor [25]. It was shown that TNFα synergizes with RANKL [36], the synergistic action being mediated through the p55TNFR [67]. In cultures of BMC, p55TNFR was found to mediate the effects of TNFα, while p75TNFR was not required for efficient osteoclastogenesis [4]. These stimulatory effects are in contrast to the findings of the present study, since we found TNFα in co-cultures of intact osteoblasts and BMC to inhibit the development of osteoclasts. The inhibition mediated by TNFα disappeared in co-cultures with *p55TNFR*$^{-/-}$ osteoblasts, p55TNFR being the only TNF receptor expressed by this cell lineage [9], and in these cultures, the number of osteoclasts was even increased. In cultures of BMC and spleen cells, however, the stimulatory effects of TNFα on the development of osteoclasts, and the dependence on the p55TNFR, could be confirmed. Stimulatory and inhibitory effects of TNFα on the development of osteoclasts were previously reported in an organ culture system for osteoclast formation [63]. Low concentrations of the factor were reported to stimulate the development of osteoclasts, while higher concentrations exerted inhibitory effects. While no mechanistic aspects were described, the data suggested complex actions of TNFα on the development of osteoclasts in dependence of concentration, timing, and hematopoietic environment.

To stimulate osteoclastogenesis via osteoblasts, TNFα was reported to modulate the expression of RANKL [2,68]. Since OPG and RANKL were described to act as crucial regulators of growth and development of osteoclast lineage cells, it has become evident that it is the ratio of the levels of the decoy receptor OPG [57] and the ligand RANKL [34] that determines the final biological activity of the factor [26]. In the present experiments, the low concentrations of TNFα (1 ng/ml) applied did not affect significantly the levels of transcripts encoding RANKL and OPG or the concentration of OPG in the cell supernatant, excluding changes of the RANKL/OPG ratio as a possible mechanism by which the cytokine mediates the inhibition of osteoclastogenesis. Furthermore, GM-CSF, which is a potent inhibitor of osteoclast development and which was previously reported to be downregulated by TNFα [56], could also be excluded as the TNFα-dependent inhibitor of osteoclast development.

While the main findings were the same for osteoblasts of *ddy* and *C57Bl/6* origin, the latter were less efficient in the support of osteoclastogenesis. Indeed, severe differences in osteoblast populations from different mouse strains have been reported with respect to their differentiation potential [14,54]. The osteoblasts from *ddy* and *C57Bl/6* strains used in the present study differed significantly in their response to 1,25(OH)$_2$D$_3$, the hormone required for osteoclastogenesis in the co-culture. Transcripts encoding ALP and RANKL were strongly induced, while OPG mRNA was repressed in *ddy* osteoblasts, and these effects were less pronounced in *C57Bl/6* cells, suggesting the production of a microenvironment more suitable to osteoclastogenesis by *ddy* osteoblasts (results not shown).

The ability of $1,25(OH)_2D_3$ to facilitate the release of the inhibitory activity by osteoblasts in response to TNFα either in co-cultures or in cultures of osteoblasts, suggests convergence of the respective signal transduction pathways, resulting in the release of the inhibitory activity. $1,25(OH)_2D_3$ induces differentiation of osteoblasts and several proteins expressed in bone contain VDREs in their promoters [64]. TNFα, on the other hand, besides its action on bone resorption, exerts inhibitory effects on bone formation. Thus, TNFα was found to inhibit the expression of ALP and the formation of nodules in primary murine bone marrow stromal cells [1]. Furthermore, TNFα inhibits binding of the VDR/RXR heterodimer to a VDRE [44] and it was demonstrated that NFκB p65 interferes with the VitD transcription complex, decreasing VitD-dependent gene transcription [39]. Other reports suggested the effects of TNFα on osteoblast differentiation being mediated through a suppressive TNFRE [38]. However, while examples for inhibition of $1,25(OH)_2D_3$ induced transcription by TNFα are well documented, until now no mechanisms have been proposed for a synergistic action of the two factors. The requirement for simultaneous signaling through the $1,25(OH)_2D_3$ and TNFα pathways is further underlined by the finding that Dex efficiently blocks the release of the inhibitory activity and did stimulate osteoclastogenesis [56]. The anti-inflammatory actions of the synthetic glucocorticoid Dex are mainly due to its interference with the NFκB signaling pathways [7,52]. The block of the TNFα induced release of an inhibitor of osteoclastogenesis by Dex demonstrates the dependence on the availability of the NFκB pathways for TNFα signal transduction. The abrogation of the TNFα induced inhibition of osteoclastogenesis by Dex may also explain in part the apparent contradiction between our data and previous reports demonstrating the stimulation of osteoclastogenesis by TNFα in co-cultures that were grown in the presence of the glucocorticoid [49,68].

A second possibility to explain the combined effects of TNFα and $1,25(OH)_2D_3$ may consist in the modulation of differentiation and/or apoptosis in osteoblast lineage cells, but so far, contradictory evidence has been presented. $1,25(OH)_2D_3$ was reported to counteract the induction of apoptosis induced by TNFα in rat osteosarcoma cells [24]. Furthermore, the suppression of differentiation of osteoblastic cells by TNFα was found to be independent of cell death [21]. Other reports described a synergistic effect of $1,25(OH)_2D_3$ and TNFα in promoting apoptosis [40,47] and it was suggested that the pro-apoptotic effect of the combined factors is caused by cell differentiation induced by $1,25(OH)_2D_3$ [47]. TNFα and $1,25(OH)_2D_3$ were also found to counteract each other's activities at the molecular level where $1,25(OH)_2D_3$ was found to regulate TNFα synthesis by bone cells [3,58], while TNFα decreased the expression levels of $1,25(OH)_2D_3$ receptors in rat osteosarcoma cells [41]. But the combination of $1,25(OH)_2D_3$ and TNFα not only affects cells of the osteoblast lineage, the two growth factors were demonstrated to be controlling myeloid differentiation and proliferation as well. Indeed, TNFα was suggested to mediate the inhibition of proliferation of myeloid precursors induced by $1,25(OH)_2D_3$ [48]. In the present study, the low concentrations of TNFα that were used for the production of the CM (1 ng/ml) were not found to affect the proliferation of *ddy* osteoblasts, but cell numbers were decreased at TNFα concentrations of 20 and 50 ng/ml (data not shown).

The effects of the CM from osteoblasts treated with TNFα/$1,25(OH)_2D_3$ were not due to TNFα carried over into the co-cultures with the CM, as was demonstrated by using inactivating anti-TNFα antibodies. While the antibodies efficiently blocked the inhibition of osteoclastogenesis in co-cultures and the release of the inhibitory activity into the medium by osteoblasts, there was no effect when the antibodies were added to the CM itself. Addition of anti-TNFα antibodies to co-cultures exerted little effects only on the development of osteoclasts. This is in contrast to other studies, where a strong inhibitory effect of anti-TNFα antibodies on the development of osteoclasts in vitro has been reported [68].

In conclusion, TNFα has been shown not only to act as a stimulator of bone resorption, but also to be able, in the absence of Dex and the presence of $1,25(OH)_2D_3$, to inhibit the development of osteoclasts. This inhibition occurs at lower concentrations than those required for the stimulation of osteoclastogenesis. The present data open the possibility that there is a difference in the effects of the growth factor whether it acts systemically on circulating precursor cells or locally on osteoblast–osteoclast interactions. It was confirmed that TNFα stimulates osteoclastogenesis through a direct effect on osteoclasts precursors, but it was also shown that TNFα, can induce osteoblasts to inhibit this process. The fact that Dex is a potent inhibitor of this effect might suggest that the inhibitory, protective, pathway may be interrupted in situations of glucocorticoid treatments, leading to an aggravation of the glucocorticoid-induced osteoporosis.

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