Although RANK-L is essential for osteoclast formation, factors such as transforming growth factor-β (TGF-β) are potent modulators of osteoclastogenic stimuli. To systematically investigate the role of TGF-β in human osteoclastogenesis, monocytes were isolated from peripheral blood by three distinct approaches, resulting in either a lymphocyte-rich, a lymphocyte-poor, or a pure osteoclast precursor (CD14-positive) cell population. In each of these osteoclast precursor populations, the effect of TGF-β on proliferation, TRAP activity, and bone resorption was investigated with respect to time and length of exposure. When using the highly pure CD14 osteoclast precursor cell population, the effect of TGF-β was strongly dependent on the stage of osteoclast maturation. When monocytes were exposed to TGF-β during the initial culture period (days 1–7), TRAP activity and bone resorption were increased by 40%, whereas the cell number was reduced by 25%. A similar decrease in cell number was observed when TGF-β was present during the entire culture period (days 1–21), but in direct contrast, TRAP activity, cell fusion, cathepsin K, and matrix metalloproteinase (MMP)-9 expression as well as bone resorption were almost completely abrogated. Moreover, we found that latent TGF-β was strongly activated by incubation with MMP-9 and suggest this to be a highly relevant mechanism for regulating osteoclast activity. To further investigate the molecular mechanism responsible for the divergent effects of continuous versus discontinuous exposure to TGF-β, we examined RANK expression and p38 MAPK activation. We found the TGF-β strongly induced p38 MAPK in monocytes, but not in mature osteoclasts, and that continuous exposure of TGF-β to monocytes down-regulated RANK expression. The current results suggest that TGF-β promotes human osteoclastogenesis in monocytes by stimulation of the p38 MAPK, whereas continuous exposure to TGF-β abrogates osteoclastogenesis through down-regulation of RANK expression and therefore attenuation of RANK-RANK-L signaling.

The skeleton is a dynamic tissue that is undergoing continuous remodeling to sustain calcium homeostasis, repair microfractures, and react to strain and stress of the skeleton. The remodeling process is a complex process and relies on the coupling between bone resorption and formation that involves osteoclasts, osteoblasts, and osteocytes. The constant regeneration of bone emphasizes the delicate balance between bone resorption and bone formation, which, if altered, may lead to pathological conditions such as osteoporosis or osteopetrosis. The investigation of the cellular actions of the major players of bone remodeling may therefore contribute significantly to the discovery of new and better drugs for the treatment of osteoporosis (1). Of particular interest for the regulation of bone turnover is the growth factors and cytokines produced in the bone microenvironment and the signal events involved in their regulation of resorption. One of many important cellular events in bone remodeling is osteoclastic bone resorption, which is preceded by osteoclastogenesis and followed by apoptosis. The life, function, and death of osteoclasts are known to be influenced at many different levels by a variety of growth factors, including but not restricted to M-CSF, vascular endothelial growth factor, parathyroid hormone, TGF-β, and tumor necrosis factor-α (2–8).

Although RANK-L is essential for osteoclast formation, factors such as TGF-β are potent modulators of osteoclastogenic stimuli (7, 9). TGF-β is by far the most abundant cytokine in bone, and by its mere abundance in bone, 200 μg/kg tissue, must be considered as a central player in bone turnover (10) and potentially able to couple bone resorption with bone formation (11, 12). TGF-β is primarily produced by the osteoblasts sequestering TGF-β in its latent form during bone formation and then subsequently released and activated when the matrix is resorbed by osteoclasts by various proteases and acid (13–16).

TGF-β is unique among growth factors in its potent and widespread actions. Almost every cell in the body has been shown to make some form of TGF-β, and almost every cell has receptors for TGF-β (14). Therefore, it becomes apparent that the mechanisms of actions of TGF-β are extensive and complex and that TGF-β possesses multifunctional biological activities. Restricted to the bone environment, target cells include osteoblasts, osteoclasts, their precursors as well as bone marrow and stromal cells (1, 10, 11).

In vivo studies of TGF-β have demonstrated its importance for bone metabolism. TGF-β1 knockout mice display an about 30% decrease in tibia length and a reduction in bone mineral content (17). In vivo studies involving exogenously administered recombinant TGF-β show that TGF-β increases bone formation and promotes fracture healing (18). Moreover, TGF-β is a part of the “vicious cycle” of developing osteolytic

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bone metastasis and giant cell tumors of bone (19–21). At the cellular level, it is well established that TGF-β both stimulates and inhibits bone formation, most likely through different actions of TGF-β at unique stages of osteoblast maturation (1, 11, 22–24, 49). The effects of TGF-β on osteoclastogenesis, bone resorption, and apoptosis are also complex, since both stimulatory and inhibitory effects in various species and culture systems have been reported (3, 9, 25–30). Since this molecule influences most if not all cells of the bone and their precursors, highly controlled and pure cell culture systems must be used when evaluating the direct molecular mechanism elicited by TGF-β. Most importantly, complex and heterogeneous cell culture systems make accurate assessment of the direct effect of TGF-β virtually impossible, since TGF-β stimulates, for example, osteoprotegerin and other factors in stromal cells that indirectly inhibit osteoclastogenesis (31, 32). Thus, since TGF-β exerts pleiotropic effects on a large range of bone cells, this has hampered the molecular understanding of the exact role of this growth factor.

The study of the direct effect of TGF-β on human osteoclastogenesis has been extremely limited because of technical difficulties with obtaining human osteoclasts in culture. However, with the recently established technique of peripheral blood monocyte purification followed by in vitro differentiation into mature osteoclasts, the possibility of investigating the direct effects of TGF-β on human osteoclastogenesis has been enabled. However, this approach results in a highly heterogeneous cell population consisting of, among other cells, T-cells, and stage of osteoclastogenesis. We find that TGF-β, for example, modulates, for example, osteoprotegerin and other factors in stromal cells that indirectly inhibit osteoclastogenesis (31, 32).

In this study, we systematically describe the effect of TGF-β on three different populations of osteoclast precursors, namely in a lymphocyte-rich, lymphocyte-poor, and pure CD14 population of cells. We describe in depth the new CD14 cell culture system for obtaining a highly pure source of human osteoclast precursors and use this specific system to assess the direct effects of TGF-β on osteoclastogenesis.

We investigate the effect of TGF-β that clearly is dependent on time and length of addition as well as both biological context and stage of osteoclastogenesis. We find that TGF-β modulates all of the osteoclastogenic phenotypes investigated (i.e. proliferation, fusion, TRAP activity, cathepsin K, and MMP-9 expression) as well as the resorptive activity itself.

By further investigation of TGF-β-controlled osteoclastogenesis, we found that TGF-β potently induced p38 MAPK and regulated RANK expression, and we therefore present data suggesting how TGF-β can modulate human osteoclastogenesis.

MATERIALS AND METHODS

Cell Culture—Isolation of CD14-positive monocytes with primary coated Dynabeads M-450 (111.1 Dynal Biotech) was as follows. Human monocytes were isolated from peripheral blood, obtained from healthy volunteers both male and female in the age range of 18–67 years. In the present studies, we did not observe differences between age and sex; however, the design was not optimized to investigate such effects.

The blood was diluted 1:1 with PBS (catalog no. BE17-512F; Bio-Whittaker). The blood/PBS was carefully layered on the on Ficoll-Paque (catalog no. 17-1440-03; Amersham Biosciences). This gradient was centrifuged at 2000 rpm for 20 min. The lymphocytes were collected from the interface between the plasma and the Ficoll-Paque and washed with ice-cold PBS followed by centrifugation at 2000 rpm for 12 min. The wash was repeated twice, after which the cells were resuspended with cold PBS containing 2% serum (catalog no. S0415; Bioreagents). The cells were kept on ice while the preparation of beads was done. For 300 million cells, 125 μl of beads (10 million beads) were used (Dynal Biotech). The tube was placed with the beads and ice-cold PBS in the magnetic device (Dynal Biotech) for 2 min, after which the supernatant was discarded. This wash was repeated three times.

Million cells were added to the beads and incubated at 4°C with end-over-end homogenization for 20 min. After the incubation at 4°C, the tube was placed in the magnetic device for 2 min, after which the supernatant was discarded. Thereafter, 5 ml of PBS containing 2% serum was added, following which the beads were gently resuspended. The tube was placed in the magnetic device for 2 min. This wash was repeated at least five times. Finally, the cells were resuspended in α-minimal essential medium containing 10% serum, 100 units/ml penicillin, 100 μg/ml streptomycin.

The cells were seeded in 75-cm² bottles and cultured in α-minimal essential medium containing 10% serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml M-CSF (catalog no. 216-MC; R&D Systems), 25 ng/ml Rank Ligand (catalog no. 310-01; Peprotech), or TRANCE (catalog no. 390-TN; R&D Systems). After 3 days, the cells were washed with PBS twice. Hereafter trypsin was added, and the cells and trypsin were incubated at 37°C for approximately 20 min. The cells were scraped off and reseeded as indicated. The cells were cultured as described above in α-minimal essential medium containing serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml M-CSF (catalog no. 216-MC; R&D Systems), and 25 ng/ml Rank Ligand (catalog no. 310-01; Peprotech) or TRANCE (catalog no. 390-TN; R&D Systems). TGF-β1 (catalog no. 100-B-010; R&D Systems) was used when indicated at 10 ng/ml.

To quantify the cell number, the AlamarBlue assay (Trek Diagnostics) was used according to the manufacturer’s instructions.

TRAP Assay—At the end of each culture period, TRAP activity was measured in the conditioned media by adding a colorimetric substrate, 6 mM p-nitrophenyl phosphate, in the presence of 25 mM sodium tartrate at pH 5.5. The reaction products were quantified by measuring optical absorbance at 405 nm.

Enzyme Assays—Human pro-MMP-2 was a kind gift from Drs. F. Frankenke and J. M. Foidart (University of Liege, Belgium). Mouse pro-MMP-9 was purified from cell culture media of transformed hamster kidney cells, by means of two consecutive chromatographic steps on gelatin-Sepharose and concanavalin A-Sepharose columns (Amersham Biosciences) (47). Pro-MMPs were activated with 4-aminophenylmercuric acetate. The molar concentram of active MMPs (48) was determined by using the MMP inhibitor BB94 (kindly provided by Dr. H. Van Wart; Roche Applied Science). Latent TGF-β was incubated with 4-aminophenylmercuric acetate-activated MMP for 5 or 24 h unless otherwise indicated in a buffer containing 50 mM Tris-Cl, 0.15 mM NaCl, 10 mM CaCl₂, 50 μM ZnSO₄, 0.05% Brij, pH 7.5). Activation of latent TGF-β was followed by Western blotting.

Bone Resorption—Bone resorption was measured by formation of resorption pits. Peripheral blood mononuclear cells and CD14-positive cells were cultured for the indicated time periods before adherent cells were scraped gently off with a cotton swab and bone slices were washed in distilled water. Resorption was assessed with Alizarin Red S hematoxylin and subsequently washed. Resorbed bone area was measured using CAST-GRID software (Microsoft Corp., Olympia, WA), and results are expressed as resorbed bone area in percentage of total bone.

The measurement of C-terminal type I collagen fragments (CTX) release from bone slices was performed by the CrossLaps for culture ELISA kit (Nordic Bioscience Diagnostics), which was used according to the manufacturer’s instructions.

Cell Fusion—Cell fusion was determined of peripheral blood mononuclear cells and differentiatd CD14-positive cells cultured for 7 days in the presence of factors as indicated. After culture, the cells were fixed in 3.7% formaldehyde and methanol, each for 5 min, and incubated in Wright solution. The slides were stained with Wright’s hematoxylin and subsequently washed. Resorbed bone area was measured using CAST-GRID software (Microsoft Corp., Olympia, WA), and results are expressed as resorbed bone area in percentage of total bone.

The measurement of C-terminal type I collagen fragments (CTX) release from bone slices was performed by the CrossLaps for culture ELISA kit (Nordic Bioscience Diagnostics), which was used according to the manufacturer’s instructions.

Western Blotting—Western blotting was performed on total cell lysates in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 0.5% NP40, 1% SDS) containing 10 mM NaF and 50 mM NaVO₄. Equal protein levels were determined and adjusted by the Bio-Rad DC protein assay according to the manufacturer’s instructions. Samples were resolved on 10% SDS-polyacrylamide gels and electrophoresed onto nitrocellulose membranes (Bio-Rad). After blotting, equal and sufficient loading was indicated (catalog no. P2096; Sigma) staining of the membranes, after which the membranes were blocked overnight at 4°C with TBS-T (50 mM Tris-HCl, pH 7.5, 0.1% Tween 20, 100 mM NaCl) containing 5% milk powder. Membranes were then incubated for 1 h at ambient temperature with antibodies against either MMP-9.
Control of Human Osteoclastogenesis by TGF-β

RESULTS

The Effect of TGF-β in a Lymphocyte-rich Environment—To systematically investigate the role of TGF-β on human osteoclastogenesis, we isolated monocytes from peripheral blood by three distinct approaches, resulting in (i) lymphocyte rich osteoclast precursor population, (ii) a lymphocyte-poor osteoclast precursor population consisting only of adherent cells, and (iii) a pure osteoclast precursor population obtained by CD14-positive magnetic cell sorting technique. In each of these populations, we investigated the effects of TGF-β on proliferation, TRAP activity, fusion, and resorption.

The lymphocyte-rich culture system is a highly heterogeneous population that contains, among other cell types, CD19 B-cells (9%), CD3 T-cells (70%), CD14 monocytes (14%), and CD56 natural killer cells (6%) (33). This heterogeneous population has previously been used to investigate the effects of TGF-β on human osteoclastogenesis (25). However, since approximately 85% of the monocyte fraction is not osteoclast precursors, the direct and indirect effects of TGF-β are impossible to distinguish. Furthermore, of the different cell types present in the peripheral blood monocyte fraction, more cells than the CD14-positive monocytes express the receptor for RANK-L, RANK. The CD19-positive B-cells have previously been reported to express RANK and to be able to differentiate into osteoclasts (33, 36–40). Furthermore, B-cell-deficient mice do not lose bone when they are ovariectomized (36). The importance of the immune system in osteoclastogenesis is further emphasized by the fact that RANK-L originally was identified on activated T-cells (41) and that T-cell-deficient mice did not lose bone when ovariectomized (42, 43). Finally, TGF-β has been proven to dramatically increase the RANK-L expression on activated T-cells (44).

Therefore, to reproduce previous published results and to expand these with the time-dependent effects of TGF-β on osteoclastogenesis, we used the described lymphocyte-rich cell population and cultured these cells in the presence of RANK-L and M-CSF (both at 25 ng/ml) and exposed the cells to TGF-β (10 ng/ml) for the indicated time periods (Fig. 1). We found, as previously described (25), that TGF-β stimulated bone resorption, namely by ~400% measured by CTX analysis (Fig. 1C) and pit area measurements (data not shown). However, resorption was only stimulated when TGF-β was present in the initial part of the culture period (Fig. 1C). When TGF-β was present for a longer time or throughout the culture period, resorption was restored to control levels (Fig. 1C). Supporting this obser-

in the presence of RANK-L and M-CSF at 25 ng/ml for the entire culture period and TGF-β at 10 ng/ml at the indicated time intervals. At the end of the culture period, the cell number was quantified by AlamarBlue and expressed as a percentage of control (A). The supernatants were collected, and TRAP activity was estimated by the TRAP assay (B). Resorption was measured in the supernatant by the Crosslaps™ ELISA (C).

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**FIG. 1.** The effect of TGF-β in a lymphocyte-rich environment. Monocytes were seeded at a density of 600,000 cells/cm² in 96-well plates on bovine cortical bone slices. Cells were cultured for 28 days (catalog no. MAB13418; Chemicon), cathepsin K (catalog no. MAB3324; Chemicon), p38 MAPK total form (catalog no. 9212; Cell signaling), p38 MAPK active form (catalog no. 9216; Cell Signaling), RANK (catalog no. AB1861; Chemicon), R-M-CSF (catalog no. AF329; R&D Systems), or TGF-β (catalog no. MAB240; R&D Systems). After washing vigorously with TBS-T for 1 h, membranes were incubated for 1 h at ambient temperature with horseradish peroxidase-conjugated antibodies (DAKO) and developed with an enhanced chemiluminescence kit (ECL™; Amersham Biosciences), according to the manufacturer’s instructions.

Statistics—All graphs show one representative experiment of at least three, each with four individual replications. All graphs show the mean of four replications and S.D. values. All statistical calculations have been performed by Student’s two-tailed unpaired t test assuming normal distribution with equal variance. Statistical significance is given by the number of asterisks (*, p < 0.05; **, p < 0.01; ***, p < 0.01.
vation, TRAP activity followed the same pattern of modulation by TGF-β (Fig. 1B). Most interestingly, proliferation was just slightly and nonsignificantly increased when TGF-β was present at the initial culture period and just slightly and nonsignificantly decreased when present through the culture period (Fig. 1A). Thus, the stimulation of bone resorption by TGF-β was not mediated by induction of proliferation of osteoclast precursors.

The Effect of TGF-β in a Lymphocyte-depleted System—The effect of TGF-β in the lymphocyte-rich cell population clearly depended on the time and length of exposure. However, when using a lymphocyte-rich culture system, it is impossible to distinguish between the direct and indirect effects of TGF-β, and we therefore used the adherent fraction to further investigate the direct effect of TGF-β on osteoclasts and their non-defined precursors.

When the lymphocyte-poor cell population was selected by allowing the cells to adhere for 3 days in the presence of M-CSF (25 ng/ml) followed by extensive washing, cell lifting by trypsin treatment, and cell scraping followed by reseeding, results diverged strongly from the results obtained with the lymphocyte-rich environment (Fig. 2, A–C).

Bone resorption was only stimulated when TGF-β was present in the beginning of the culture periods, albeit to a much lesser extent (approximately 40% measured by CTX analysis) (Fig. 2C) and pit area measurements (data not shown) when compared with the lymphocyte-rich cell population. TRAP activity was not stimulated (Fig. 2B), and the cell number was significantly inhibited when TGF-β was present for longer time periods than the initial culture period (Fig. 1A).

These results clearly demonstrate the utmost importance of defining the culture system and conditions and show that for the larger part the stimulatory effects of TGF-β are restricted to an indirect effect from the lymphocyte fraction of the cells. However, resorption was significantly stimulated in the adherent cell population when TGF-β was present in the initial part of the culture period, strongly indicating that TGF-β asserts effects by both direct and indirect mechanisms.

We conclude from the experiment made in the lymphocyte-rich and -poor cell populations that, for the larger part, TGF-β is either counteracting negatively signals from the lymphocyte population, as suggested by Ref. 25, or promoting RANK-L expressing in other cells than osteoclasts precursors (36), thereby asserting the effects through indirect interactions.

However, when using only the adherent population, the 40% increase in resorption when cells were exposed to TGF-β in the initial culture period is highly interesting for understanding the direct effect of TGF-β on osteoclast precursors. We therefore further developed the culture system as described below.

Establishment of a Pure Human Osteoclast Precursor Cell System—To further investigate the direct effects of TGF-β on osteoclast precursors, we isolated CD14-positive cells from the lymphocyte-rich fraction by a CD14 magnetic bead isolation technique.

To assess the morphological characteristics of these cultures, we cultured monocytes isolated by CD14 magnetic bead isolation for 14 days in the presence of either M-CSF (Fig. 3A) or

![Fig. 2. The effect of TGF-β in a lymphocyte-depleted system.](image-url)

For selection of only adhered cells, cells were grown for 3 days in the presence of 25 ng/ml M-CSF followed by detachment by trypsin treatment. The cells were reseeded at a density of 350,000/cm² in 96-well plates on bovine cortical bone slices. Cells were cultured for 28 days in the presence of RANK-L and M-CSF at 25 ng/ml for the entire culture period and TGF-β at 10 ng/ml at the indicated time intervals. At the end of the culture period, the cell number was quantified by AlamarBlue and expressed as a percentage of control (A). The supernatants were collected, and TRAP activity was estimated by the TRAP assay (B). Resorption was measured in the supernatant by the Crosslaps™ ELISA (C).
We assessed bone resorption and found a dose-response relationship between the concentrations of RANK-L and pit area, with an EC_{50} concentration of approximately 15 ng/ml (Fig. 4E).

We assessed resorption as a function of time by the Cross-Laps assay, where conditioned medium was harvested from the same wells every third day, and found that resorption nicely correlated with the appearance of large multinucleated osteoclasts at day 11, at which time TRAP activity was evident (Fig. 4F). Moreover, the resorption was directly proportional to the length of the culture period.

When using the cell concentration of 100,000 cells/cm², the different stages of osteoclastogenesis based on the parameters of cell fusion, TRAP activity, cathepsin K expression, and resorption presented in Fig. 3, A and B, and Fig. 4, A–F, is estimated as follows: monocyte characteristics, absence of fusion and TRAP activity from day 0 to 6; preosteoclast characteristics, low levels of fusion, TRAP activity, and cathepsin K expression from day 6 to 8; mature osteoclast characteristics, multinucleation, bone resorption, high TRAP activity, and high cathepsin K expression from day 9 and following.

The Direct Effects of TGF-β on Pure (CD14) Osteoclast Precursors—To further investigate the effects of TGF-β on pure osteoclast precursors, we used the CD14-positive magnetic cell sorting monocyte preparations as described above.

CD14-positive cells were exposed to TGF-β at different time intervals as indicated in Fig. 5, A–C. At the end of the culture period, proliferation, TRAP, and resorption were investigated (Fig. 5, A–C).

When monocytes were treated with TGF-β in the beginning of the culture periods, we observed in conjunction with the adherent cell population a 40% increase in bone resorption (Fig. 5C). This was accompanied by an approximately 75% increase in TRAP activity (Fig. 5B).

Most interestingly, TGF-β almost completely abrogated bone resorption measured by CTX release (Fig. 5C) and pit area (data not shown) when present throughout the culture period. TRAP activity followed the same pattern of modulation by TGF-β as resorption (Fig. 5B).

The cell number was significantly reduced when TGF-β was present in early, middle, and late periods of the culture period (Fig. 5A). When TGF-β was exposed to osteoclast precursors and mature osteoclasts, TGF-β inhibited bone resorption significantly (Fig. 5C).

Therefore, the effect of TGF-β is highly dependent on time and length of the addition. This strongly emphasizes the cell differentiation stage-specific effects of TGF-β on human osteoclastogenesis, since it can both promote and inhibit TRAP activity and bone resorption.

TGF-β Controls Osteoclastogenesis by Blocking Fusion of Precursors and TRAP Activity—To further investigate the effect of TGF-β on osteoclastogenesis, we examined cell fusion and TRAP activity of osteoclast when CD14 magnetic cell sorting monocytes were continuously exposed to TGF-β.

As shown in Fig. 5, C and D, TGF-β strongly inhibited TRAP activity and the bone resorbing when present during the entire culture period. To further understand the direct effects of TGF-β on this well defined precursor population of osteoclasts, we investigated the morphology of the CD14 isolated cells treated with TGF-β plus M-CSF and RANK-L.

When cultured on plastic to assess osteoclast cell fusion by Wright-Giemsa staining, TGF-β completely prevented the fusion of osteoclast precursors (Fig. 6C). Accordingly, TRAP activity was completely abrogated in the presence of TGF-β, RANK-L, and M-CSF (Fig. 6B). Cell proliferation was inhibited in all conditions by the treatment of TGF-β.

To further describe this cell culture system, we assessed osteoclastogenesis by monitoring fusion, TRAP activity, and bone resorption in the presence of either M-CSF or M-CSF plus RANK-L at 2–11 days and assessed cathepsin K expression by Western blotting (Fig. 3C). Cathepsin K was only detectable in the presence of RANK-L, emphasizing the purity and robustness of this system.

To further describe this cell culture system, we assessed osteoclastogenesis by monitoring fusion, TRAP activity, and bone resorption in the presence of either M-CSF or M-CSF plus RANK-L at five different cell concentrations (Fig. 4, A–D).

We found that cell fusion only was present when RANK-L was added (Fig. 4, A and B), in contrast to the lymphocyte-rich environment, where fusion events are ongoing in the absence of exogenous RANK-L (data not shown). TRAP activity was induced more than 50-fold in the presence of RANK-L (Fig. 4, C and D).

The optimal CD14 cell concentration was estimated to be 100,000 cells/cm² and was used for all of the following experiments with TGF-β. At this specific cell concentration, fusion was absent at day 6, strongly in progress at day 8, and with large multinucleated osteoclasts at day 10. TRAP activity was absent at day 6 and developed in the same manner as fusion (i.e. absent at day 6 and strongly augmented from day 6 to 10 of culture).

M-CSF plus RANK-L (Fig. 3B), followed by TRAP stainings. Neither TRAP-positive cells nor multinucleated cells were observed in the M-CSF-treated condition. This is in contrast to the highly heterogeneous cultured system consisting of the crude monocyte population, where both higher levels of TRAP and fusion are observed in the absence of exogenous added RANK-L, suggesting RANK-L to be produced in the culture by various cells (data not shown). When treated with both M-CSF and RANK-L, a highly homogeneous population of large multinucleated TRAP positive cells was formed (Fig. 3B).

To further investigate the CD14 cells, we cultured CD-14-positive monocytes in the presence of either M-CSF or M-CSF plus RANK-L for 2–11 days and assessed cathepsin K expression by Western blotting (Fig. 3C). Cathepsin K was only detectable in the presence of RANK-L, emphasizing the purity and robustness of this system.

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FIG. 4. Dynamic assessment of human osteoclastogenesis in a pure osteoclast precursor (CD14) cell system. CD14-positive cells were isolated with the Dynal Biotech magnetic bead isolation procedure. The cells were reseeded at 15,000, 30,000, 65,000, 100,000, or 165,000 cells/cm² and cultured for 16 days with refreshment of media containing 25 ng/ml M-CSF (A and C) or 25 ng/ml M-CSF and RANK-L (B and D) and collection of supernatants every second day. At each time point, one plate was fixed in 5% formaldehyde, followed by investigation of fusion index by Wright-Giemsa staining. A and B, fusion evaluated by Wright-Giemsa staining. C and D, TRAP activity in the harvested supernatants. E, 350,000 CD14-positive cells/cm² were seeded on bovine bone slices cultured with M-CSF (25 ng/ml) and various concentrations of RANK-L as indicated.
Therefore, we conclude that continuous exposure of osteoclast precursors to TGF-β inhibits differentiation of monocytes into osteoclasts. This is to our knowledge the first description of TGF-β modulating osteoclastogenesis to such an extent as described above.

The Stimulatory Effect of TGF-β on Osteoclastogenesis Is Restricted to Monocytes—To further investigate the apparent contrast between stimulation of osteoclastogenesis in monocytes and abrogation of osteoclastogenesis in preosteoclasts when exposed to TGF-β, we cultured CD14 cells with M-CSF and RANK-L and exposed the cells to TGF-β at various time points. Cell number, TRAP activity, and cell fusion and was measured at the end of the 10-day culture period (Fig. 7, A–C).

When TGF-β was present at the monocyte stage of the culture (days 1–6), TRAP activity was significantly increased. When TGF-β was present throughout the culture period (days 1–10) or at the stage of preosteoclasts (days 6–10), both cell fusion and TRAP activity were strongly inhibited by more than 75%.

The number of multinucleated osteoclasts at the end of the culture period (day 10) was approximately the same when TGF-β was present at the monocyte stage of differentiation (Fig. 6C). Thus, initial exposure of monocytes to TGF-β followed by discontinuation before the preosteoclasts level does not block fusion. For verification, as presented in Fig. 7, D–F, by Wright-Giemsa stainings, at day 10 of differentiation, CD14 cells treated with either M-CSF (D) or M-CSF plus RANK-L supplemented with TGF-β at days 6–10 (F) were mononuclear still with magnetic beads visible, as quantified in Fig. 6C. As expected, cells cultured with M-CSF plus RANK-L (E) were multinucleated with osteoclast characteristics and did not have beads attached.

These experiments pinpointed the stage of differentiation at which the effect of TGF-β is shifted from promoting to counteracting osteoclastogenesis. Namely, TGF-β asserts a positive effect on monocytes and a negative effect on preosteoclasts and mature osteoclasts.

TGF-β Controls Protease Expression—To further investigate the effects of TGF-β on monocyte differentiation and to clarify the TGF-β-promoted phenotype, we assessed the protein expression of cathespin K and MMP-9 by Western blotting in cells treated with M-CSF, M-CSF plus RANK-L, or M-CSF plus RANK-L and TGF-β (Fig. 8).

In complete alignment with the previous results, TGF-β treatment blocked the expression of cathespin K (Fig. 8A). Furthermore, MMP-9 expression was strongly attenuated (Fig. 8D).

MMP-9 Activates Latent TGF-β—TGF-β is primarily produced by the osteoblasts sequestering TGF-β in its latent form during bone formation. TGF-β is then subsequently released and activated when the matrix is resorbed by osteoclasts through secretion of protons and by various proteases (13–16).

To further investigate the role of TGF-β in osteclast biology, we incubated latent TGF-β with the osteoblastic MMP-2 and the osteoclastic MMP-9 for 5 or 24 h and analyzed activation by Western blotting. Incubation of latent TGF-β with MMP-9 resulted in an increase in the intensity of a protein band at 25 kDa, corresponding to activated TGF-β (1, 24) already after 5 h of incubation and even more prominently at 24 h (Fig. 9). In the presence of the general MMP inhibitor, BB-94, the increase of this signal at 25 kDa was totally abrogated, thus confirming an MMP-dependent activation of latent TGF-β (Fig. 10). Most interestingly, only the osteoclastic MMP-9 and not the osteoblastic MMP-2 was able to activate TGF-β.

Since active TGF-β is down-regulating the MMP-9 activity in osteoclasts (Fig. 8) and MMP-9 has the ability to activate latent TGF-β, this constitutes a possibility for a highly relevant feedback mechanism controlling osteoclast activity.

TGF-β Stimulates Osteoclastogenesis through the p38 MAPK—The p38 MAPK is a downstream target of the RANK pathway in osteoclasts (60) and has previously been shown to be essential for osteoclastogenesis (59–61). Therefore, to further investigate the molecular mechanism involved in TGF-β-controlled osteoclastogenesis, we cultured monocytes and mature osteoclasts to investigate whether an augmentation of p38 MAPK activity was seen immediately after TGF-β exposure. As presented in Fig. 10, TGF-β strongly augmented p38 MAPK activity in monocytes in a time-dependent manner but not in mature osteoclasts.

Most interestingly, we did not detect active p38 MAPK in mature osteoclasts. Moreover, we did not detect an induction of the p38 MAPK by TGF-β. Only in one single experiment did we observe a small background level of active p38 MAPK in mature osteoclast, however to a much lower extent than induced or background levels in monocytes (data not shown).

This is in direct alignment with the previously finding establishing a role of the p38 MAPK in osteoclastogenesis but not in mature osteoclasts by use of the p38 MAPK-specific inhibitor SB203580 (59).

Thus, these results further corroborate the importance of the p38 MAPK in osteoclastogenesis and not in mature osteoclast function and suggest that TGF-β plays a role in osteoclastogenesis by further activating the p38 MAPK in monocytes but not in mature osteoclasts.

TGF-β Regulates RANK Expression—Previously, TGF-β has been shown to regulate RANK RNA expression (27). Exposure of mouse RAW 264.7 cells to TGF-β led to an argumentation of RANK expression by −3-fold (27). Therefore, to further mechanistically explain the divergent effect of TGF-β on human osteoclastogenesis, we investigated the protein expression of RANK and the receptor for M-CSF (R-M-CSF) expression when monocytes continuously exposed to TGF-β compared with RANK-L and TGF-β conditions.

As seen in Fig. 11, exposure of TGF-β to monocytes grown for 12 days strongly attenuated RANK expression but not R-M-CSF. Thus, the current divergent results of TGF-β are in part explained by the fact that TGF-β initially is inducing osteoclastogenesis through the p38 MAPK but through continuous exposure strongly down-regulates RANK expression and therefore attenuates osteoclastogenesis because of attenuated RANK-L signaling.

DISCUSSION

Since growth factors such as TGF-β and tumor necrosis factor-α often are associated with pathological bone turnover, downstream targets in osteoclasts could be potential targets for drugs counteracting the increased osteoclastogenesis observed in pathological situations.

The effects of TGF-β have heretofore not been systematically investigated in primary human monocytes. In this study, we wanted to fully elucidate the direct effects of TGF-β on human osteoclastogenesis in vitro for later use in genomics approaches.
FIG. 5. The direct effects of TGF-β on pure (CD14) osteoclast precursors. CD14-positive cells were isolated with the Dynal Biotech magnetic bead isolation procedure. The cells were reseeded at a density of 350,000 cells/cm² in 96-well plates on bovine cortical bone slices. The cells were cultured for 21 days in the presence of RANK-L and M-CSF at 25 ng/ml for the entire culture period and TGF-β at 10 ng/ml at the indicated time intervals. At the end of the culture period, the supernatants were collected. Cell number was quantified with AlamarBlue and expressed as a percentage of control (A). TRAP activity was investigated by the TRAP assay in the supernatant (B). Bone resorption was measured in the supernatant by the Crosslaps™ ELISA (C).

FIG. 6. TGF-β controls osteoclastogenesis by blocking fusion of precursors and TRAP activity. CD14-positive cells were isolated with the Dynal Biotech magnetic bead isolation procedure. The cells were seeded at a density of 100,000 cells/cm² in 96-well plates, and cells were cultured for 11 days in the presence of 25 ng/ml M-CSF, 25 ng/ml M-CSF plus RANK-L, or 25 ng/ml M-CSF + RANK-L and supplemented with TGF-β at 10 ng/ml. At days 7, 9, and 11, cell number was measured by AlamarBlue and expressed as a percentage of M-CSF-treated cells (A) followed by fixation in 5% formaldehyde for investigation of cell fusion by Wright-Giemsa (C). TRAP activity was investigated by the TRAP assay in the supernatant (B).
for discovering unique targets in osteoclasts that by pharmaceutical regulation would reduce pathological bone resorption in, for example, osteoporosis and bone metastatic cancer.

Therefore, to fully understand the role of TGF-β on human osteoclastogenesis and to compare our own results with previously published results, we have used three different models of human osteoclastogenesis and investigated the osteoclast parameters of proliferation, TRAP activity, fusion, MMP-9 and cathepsin K expression, and resorption with regard to time and length of exposure to TGF-β.

By using magnetic cell sorting CD14-positive cells, we have described the direct effect of TGF-β on human osteoclastogen-
The control of human osteoclastogenesis by TGF-β

FIG. 8. TGF-β controls protease expression. CD14-positive cells were isolated with the Dynal Biotech magnetic bead isolation procedure. The cells were seeded at a density of 100,000 cells/cm² in 75-cm² flasks and cultured in the presence of either 25 ng/ml M-CSF, M-CSF plus RANK-L at 25 ng/ml, or M-CSF plus RANK-L at 25 ng/ml supplemented with 10 ng/ml TGF-β for 12 days. At the end of the culture period, cells were washed and lysed in radioimmune precipitation assay buffer for analysis by Western blotting for cathepsin K expression (A) and MMP-9 expression (B).

FIG. 9. MMP-9 activates latent TGF-β. The activation of latent TGF-β was analyzed by Western blotting under nonreducing conditions. A significant increase of 40% in resorption was observed. However, when continuous exposure to TGF-β was sustained, this completely blocked both resorption and TRAP activity.

We found that TGF-β blocked fusion and TRAP activity when present throughout the culture, and we pinpointed the stimulatory effect of TGF-β to be restricted to the monocytes. When TGF-β was added to preosteoclasts, cell fusion, TRAP activity, and later resorption were potently inhibited.

We clearly demonstrate that TGF-β strongly inhibits cathepsin K and MMP-9 expression in monocytes treated with M-CSF and RANK-L, thus inhibiting most parameters of osteoclastogenesis.

By further investigation of the molecular mechanism responsible for the divergent effects of TGF-β on osteoclastogenesis, we show that TGF-β strongly arguments p38 MAPK activity in monocytes but not in mature osteoclasts, which previously have been shown to be essential for osteoclastogenesis. Furthermore, continuous exposure of monocytes to TGF-β almost abrogates RANK expression, suggesting that attenuated RANK signaling is responsible for the abrogation of osteoclastogenesis by continuous TGF-β exposure. Therefore, these results begin to explain the divergent effects of TGF-β observed on human osteoclastogenesis.

We have reproduced the previous findings of the stimulatory effects of TGF-β in a lymphocyte-rich environment and shown this effect to be restricted to the initial culture period. In contrast, when TGF-β was present throughout the culture period, no stimulatory effects were seen on either bone resorption or TRAP activity. When using only the adherent cells of the lymphocyte-rich population, similar characteristics were observed, however only with a minutely stimulatory effect in the initial culture period.

Thus, by systematically investigating the effect of TGF-β in three different culture systems, we clearly demonstrate that the presence of TGF-β at a given stage of human osteoclastogenesis dramatically influences several steps of this process (i.e., cell proliferation, TRAP expression, RANK expression, cell fusion, and protease expression) and modulates strongly the bone resorptive activity itself.

When addressing the literature for the effects of TGF-β on osteoclastogenesis in vitro, the common denominator is that TGF-β promotes osteoclastogenesis in rat and mouse oste-
oclasts, disregarding the interval of exposure to TGF-β, in contrast to the present results showing both positive and negative effects on human osteoclastogenesis.

The effect of TGF-β on osteoclastogenesis has previously been evaluated in mice by various techniques for obtaining osteoclast precursors. TGF-β promotes osteoclastogenesis when present throughout the cultures in mouse RAW and mouse spleen cell cultures (9) (TGF-β was used at 10 ng/ml). Furthermore, TGF-β stimulated TRAP-positive cells when added both early and late in the raw mouse cultures but only when added early in mouse spleen cultures (9).

When culturing mouse unfractionated bone cells, including both stromal cells and osteoclast precursors originating from mouse bone marrow, neutralizing antibodies to TGF-β abolished osteoclastogenesis assessed by cell fusion (3). Furthermore, TGF-β present throughout the culture period strongly augmented the cell fusion (3) (TGF-β was used at 10 ng/ml). When TGF-β was present throughout the culture period in both cultures of mouse mononuclear cells isolated from peripheral blood and mouse bone marrow cells, TGF-β (1 and 10 ng/ml) strongly augmented osteoclastogenesis (46). Furthermore, in these cultures, it was shown that a soluble receptor to TGF-β (TGF-βRII) inhibited osteoclastogenesis assessed by resorption (46).

Hitherto, only one paper has investigated the effect of TGF-β on human osteoclastogenesis, using a lymphocyte-rich and -poor cell culture. When TGF-β was present initially for 4 days, resorption was augmented in a lymphocyte-rich environment but not in a lymphocyte-poor environment (25). TGF-β restored the resorption levels in the lymphocyte-rich environment to the levels of the lymphocyte-poor environment. It was speculated that TGF-β could down-regulate negatively factors coming from the lymphocytes affecting osteoclastogenesis (25), which is in alignment with the current results. Taken together, the effects of TGF-β clearly depend on the cell culture system in question and most importantly the species under investigation.

In contrast to mice in which TGF-β seem to be overall stimulating, the role in human cells is more complicated, with both stimulatory effects on monocytes and inhibitory effects on preosteoclasts and mature osteoclasts.

The effect of TGF-β on the individual cells in the bone microenvironment depended on a number of parameters such as concentration, level of maturation, and time and length of exposure. Therefore, when evaluating the present results in the context of the bone microenvironment in vitro, the effects of TGF-β on osteoclasts, osteoblasts, and osteocytes must be taken into account.

TGF-β is primarily produced by osteoblasts in the latent form and sequestered in the bone matrix during bone formation. Latent TGF-β is then subsequently released and activated during resorption or even produced by the osteoclasts (13–16).

In the present experiments, the stimulatory effects of TGF-β were restricted to the level of monocytes and strongly inhibiting both preosteoclast and mature osteoclast differentiation and function. Therefore, when TGF-β is released during osteoclast resorption, the present results strongly suggest that TGF-β has a negative effect on mature osteoclasts; thus, TGF-β could be controlling the depth of the resorption lacunae and therefore the amount of resorption by the mature osteoclasts. Thus, TGF-β potentially acts as negative feedback by controlling the amount of resorptive material after activation by, among other proteases, MMP-9.

The positive effect of TGF-β on human osteoclastogenesis is restricted to the level of monocytes and is limited compared with the negative effect on preosteoclasts and mature osteoclasts. However, since osteoclasts are terminally differenti-ated cells with a restricted life span, these cells must be continually replaced in the leading edge of a haversian channel (50–52). Therefore, factors recruiting and promoting monocytes to preosteoclasts must continually be secreted, and TGF-β would be a strong candidate for this event. Supporting this, in pathological bone turnover as in the case of bone metastasis, TGF-β has been associated with first the chemotaxis of cancer cells to the resorption sites (73, 76) and subsequently the increased number of osteoclasts surrounding the cancer cells in the bone metastasis (19–21, 53). This could be a result of both the positive effect on recruitment of monocyte and osteoclast precursors to the future sites of resorption (28, 65, 72, 73, 75) and, as just as importantly, an integral part of the “viscous cycle” in which osteoclasts and the cancer cells engage in symbiosis by releasing paracrine stimulatory molecules (19–21, 53). Supporting the first hypothesis, a positive effect on chemotaxis in the response to TGF-β is observed in a still increasing amount of studies (28, 45, 65, 67, 72–78). The chemotaxis effects of TGF-β have been studied in, among other cells, monocytes, osteoblasts, osteoclasts, and cancer cells and found to be highly stimulatory, possibly through the p38 MAPK signaling pathway (77), which TGF-β on several occasions has been shown to potently induce in a number of bone cell types (11, 77). Taken together, this strongly suggests that TGF-β is recruiting new osteoclasts to the future site of resorption, thereby ensuring a continuously relieve of these terminal differentiated cells.

The TGF-β released from the bone has different effects on osteoblasts as well as osteoclasts, depending on their level of maturation. In normal bone turnover, TGF-β has been suggested to be important for the chemotaxis of new osteoblasts to the site of resorption in order to start synthesizing new bone (79). At the same time, TGF-β is stimulating osteoclast proliferation, blocking mature osteoblast proliferation, and initiating bone formation, and finally at the late stage of bone formation, TGF-β promotes osteocyte survival (1, 11, 22–24, 29). Thus, the released and synthesized TGF-β from osteoclasts affects the entire cell population of the bone, both precursors and their mature counterparts, and TGF-β is therefore potentially coupling bone resorption to bone formation.

The interplay between osteoclasts, osteoblasts, and osteocytes partly explains the contradictory findings made in different transgenic mice with regard to TGF-β (49, 55, 56). These apparent contradictions in the attempts to dissect the TGF-β signal in bone in vivo have been attributed to differences in its temporal and spatial expression (55) and highlight the need for simple in vitro systems for understanding the direct effect of TGF-β on each individual cell type with regard to time and length of exposure.

Osteoclastogenesis and osteoclast function have in recent years been extensively investigated with regard to intracellular signaling mechanisms. Several publications have found a number of kinases and coupling factors to be important in osteoclast function. Among these are TRAF6, c-Src, Pyk2, c-Cbl, NF-κB, Akt, and the MAPKs p38, p44/42, c-Jun N-terminal kinase, and SOCS-3 (2, 57–64, 66, 67–70). In the current study, we show that TGF-β potently activates the p38 MAPK in monocytes but not in mature osteoclasts. Since TGF-β has been shown to be a powerful activator of the p38 MAPK in other cell types (11, 77) and to be essential for osteoclastogenesis (60, 61), since TGF-β-induced chemotaxis was blocked by an inhibitor of the p38 MAPK (77), and last since TGF-β has been suggested to play an important role in the commitment of precursors to osteoclast differentiation (71), this is in direct alignment with the current results on monocytes. This strongly implies that the p38 MAPK is playing an extremely important role in the recruitment of
new monocytes to the future site of resorption and later osteoclastogenesis, which is augmented in the current study by TGF-β exposure to monocytes.

By further investigation of the RANK RANK-L signaling pathway in conjunction with TGF-β, we found that continuous TGF-β exposure to monocytes inhibits RANK-L signaling, which has been shown to be essential for osteoclastogenesis (58).

Previously, TGF-β has been shown to augment RANK RNA expression in the RAW 264.7 mouse cell lines at both the level of monocytes and mature osteoclasts (27). This is in complete conjunction with the overall stimulating effects of TGF-β in rodent systems, in contrast to the more complex and divergent roles of TGF-β in human osteoclasts (59). This further highlights the difference between the rodent and human systems, namely that in the mouse RAW cells TGF-β is overall stimulating not dependent on either time or length of TGF-β exposure, whereas the current results with human cells strongly indicate that TGF-β exerts different effects depending on both the level of maturation of the monocytes and time and length of exposure.

Further investigations of the complex interplay of the molecules mentioned above and others will hopefully provide further insight into the molecular mechanism of TGF-β-controlled human osteoclastogenesis and help to further understand the results presented in this study. However, as shown in the current studies, the level of maturation, the cell type, and species investigated must receive utmost attention to fully understand the effect of TGF-β and its role in human osteoclastogenesis and function.

In conclusion, we suggest that TGF-β is activated by MMP-9 of the osteoclast origin. This enables TGF-β to act as a negative feedback on mature osteoclastic resorption, controlling the volume of excavated pits by down-regulating RANK expression, and at the same time recruit new monocytes to the future site of resorption through stimulation of the p38 MAPK. Thus, TGF-β plays an important role in bone turnover, modulating human osteoclastogenesis and bone resorption.
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