Ligand-induced downregulation of receptors for TGF- β in human osteoblast-like cells from adult donors

J Gebken³, A Feydt³, J Brinckmann², H Notbohm³, P K Müller³
and B Bätge¹

¹Medizinische Klinik I, Medizinische Universität zu Lübeck, Ratzeburger Alle 160, D-23538 Lübeck, Germany

²Klinik für Dermatologie, Medizinische Universität zu Lübeck, Ratzeburger Alle 160, D-23538 Lübeck, Germany

³Institut für Medizinische Molekularbiologie, Medizinische Universität zu Lübeck, Ratzeburger Alle 160, D-23538 Lübeck, Germany

(Requests for offprints should be addressed to B Bätge)

Abstract

High concentrations of transforming growth factor β (TGF- β) are found in the bone matrix, reflecting a pivotal role of this growth factor in the coupling of bone resorption and formation. TGF- β strongly stimulates the synthesis of extracellular matrix proteins, but *in vitro* studies show an inhibitory effect on the final mineralization process, which *in vivo* occurs despite high concentrations of TGF- β . Little is known about how bone-forming cells respond to different concentrations of TGF- β and if they can transiently adapt receptor numbers in order to modulate cellular activity. Against this background, we studied the cell-surface expression of TGF- β receptors (T β R) I, II and III (betaglycan) on human osteoblast-like cells from adult donors, and examined the T β R presentation on these cells after a preceding exposure to TGF- β 1. Affinity crosslinking studies with disuccinimidylsuberate showed the presence of all three receptor types. Preincubation with TGF- β 1 markedly reduced ¹²⁵I-TGF- β 1 binding in a time-dependent and dose-dependent manner and

revealed a 95% reduction after an 18-h preincubation with 200 pM TGF- β 1. In parallel, Scatchard analysis showed that the binding affinity did not change as a consequence of TGF- β 1 preincubation. Immunoblotting analyses revealed an almost complete disappearance of immunoreactive T β R-II and T β R-III proteins after a 24-h preincubation with TGF- β 1. Using semi-quantitative reverse transcription PCR, no effect of TGF- β 1 on the expression of T β R-II mRNA was observed. These studies demonstrate a ligand-induced downregulation of T β Rs-II and -III on human osteoblast-like cells, without any evidence for recovery within the first 24 h, both in the presence and after the removal of the ligand. The underlying mechanism appears to be based on post-transcriptional events. The results suggest that high concentrations of active TGF- β 1 decrease the responsiveness of osteoblasts towards this growth factor.

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Introduction

Transforming growth factors β (TGF- β s), together with insulin-like growth factors I and II, are the most abundant growth factors stored in bone and known to function as potent regulators of osteoblast proliferation and differentiation and of production of extracellular matrix proteins (Bonewald & Dallos 1994, Centrella *et al.* 1994). In particular, these factors exert a strong influence on the quantity and quality of collagen synthesized by human osteoblasts *in vitro* (Centrella *et al.* 1992, Seitzer *et al.* 1995). The biological effects of TGF- β are mediated by TGF- β receptors types I and II (T β R-I and -II), which form a hetero-oligomeric signalling complex (Yamashita *et al.* 1994). Whereas both these receptors, but not betaglycan (T β R-III), are required for inducing signal transduction, only T β R-II can bind the ligand even in the absence of T β R-I, but not *vice versa* (Franzén *et al.* 1993).

As TGF- β has been shown to prevent the development of differentiated phenotypes – for example those associated with mineralization – changing biological effects of TGF- β in different stages of tissue formation are of central importance (Wrana *et al.* 1988, Noda 1989, Harris *et al.* 1994). TGF- β is secreted by bone cells and stored in the bone matrix in a latent form, ready to be activated and inactivated at key, pivotal stages in the process of bone formation (Bonewald & Dallas 1994). There is recent experimental evidence that activated TGF- β can be extracted in substantial concentrations from the bone matrix (Pfeilschifter *et al.* 1998). However, little is known about how the responsiveness of osteoblasts towards active TGF- β present in the osteoblastic microenvironment might be regulated in humans, although adaptive changes of the responsiveness might represent further control mechanisms once TGF- β is activated. Changing biological effects of TGF- β as a result of altered receptor binding

under the influence of certain growth factors have been demonstrated by *in vitro* studies using non-human or transformed bone cells (Centrella *et al.* 1995, 1988, Iimura *et al.* 1994). Interaction with matrix collagen was shown to be able to reduce the number of cell-surface receptors and actions of TGF- β in murine osteoblastic cells (Takeuchi *et al.* 1996). Furthermore, betaglycan (T β R-III), which is seemingly not involved in the regular signal transduction pathway, may modulate TGF- β -induced responses of mesenchymal cells. Accordingly, there is circumstantial evidence that betaglycan in its soluble form might act as a potential antagonist of TGF- β , whereas in a membrane-associated form it may enhance the binding of TGF- β to the signalling receptors I and II (López-Casillas *et al.* 1994, Massagué *et al.* 1994). Other regulatory mechanisms are suggested by a set of experiments which show that a rapid turnover of T β Rs -I, -II and -III by ligand-independent and ligand-dependent mechanisms are observed in non-human, fetal cell culture systems (Centrella *et al.* 1996). The present study was undertaken to investigate the influence of TGF- β 1 on the cell-surface expression of its own receptors in cultured human osteoblast-like cells from adult donors. Special emphasis was placed upon T β R-II, as it is important for the initial binding of TGF- β and triggers the cytoplasmic signalling cascade associated with T β R-I.

Materials and Methods

Human bone cell culture

Human osteoblast-like cells (hOBs) were isolated from the trabecular bone of adult femoral head samples obtained by informed consent during routine hip replacement surgery, as described previously (Seitzer *et al.* 1995). Briefly, fragments were seeded as explants into tissue culture flasks and cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. The cells reached confluency after 3–4 weeks. Culture medium was Dulbecco's modified Eagle's medium supplemented with 50 mg/ml L-ascorbate, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 10% fetal calf serum (FCS). Cells were defined as osteoblast-like cells by the determination of osteoblast markers: cytochemical staining for alkaline phosphatase (ALP) could be demonstrated on 60–80% of the cells and was negative on control fibroblasts; the ALP activity was stimulated 3.7-fold by 1,25dihydroxy vitamin D₃ (50 nM), and the osteocalcin concentration increased 18-fold under the same conditions; incubation with 90 nM human parathyroid hormone(1–34) stimulated cAMP fourfold; *in vitro* mineralization could be demonstrated (Seitzer *et al.* 1995). In addition, the human osteosarcoma-derived osteoblastic cell line, MG63, was used for the crosslinking experiments.

Incubation conditions

In order to analyse the influence of TGF- β 1 on cell-surface binding of ¹²⁵I-TGF- β 1 to specific receptors on hOB, three independent experiments with double determinations each were performed in different cell populations by adding TGF- β 1 for 0.5, 2, 4, 8, 18 and 24 h in three different concentrations (0, 40 and 200 pM) before the analyses described below. Before the incubation medium was removed, cells were placed on ice to preserve the effects of TGF- β 1. Alternatively, TGF- β 1 was removed after an 18-h preincubation in order to investigate receptor recovery in the absence of the ligand. Control experiments using ¹²⁵I-TGF- β 1 (40 pM) instead of native TGF- β 1 were performed according to the procedure of Massagué & Like (1985), to rule out any residual occupancy of cellular receptors for TGF- β after the first incubation.

Binding assay

Binding studies were performed according to the technique of Massagué (1985), using ¹²⁵I-labelled TGF- β 1 obtained in a lyophilized form (Amersham-Buchler, Braunschweig, FRG), and reconstituted in 100 ml 4 mM HCl, 2 mg/ml BSA and diluted with binding buffer (128 mM NaCl, 5 mM KCl, 5 mM MgSO₄, 1.2 mM CaCl₂, 50 mM HEPES, pH 7.5, 2 mg/ml BSA) to a 100 pM stock solution. Suitable aliquots were stored at –80 °C until required for use. All cell culture dishes and tubes used for analysing TGF- β 1 binding were of polypropylene or siliconized material.

To prepare cultures for binding studies, cells were seeded into six-well dishes and grown for 24 h. The medium was removed and confluent monolayer cultures of cells were washed with 1 ml binding buffer and allowed to equilibrate with binding buffer at 4 °C. After 30 min, the buffer solution was aspirated from the plates on ice. Ice-cold binding buffer and ¹²⁵I-TGF- β 1 were added to different concentrations of ¹²⁵I-TGF- β 1 in the range 1.25–30 pM and to a final volume of 2 ml. The cells were then incubated at 4 °C on a platform oscillating at 120 r.p.m. for 3.5 h. In addition, a 50-fold excess of unlabelled TGF- β 1 added immediately before incubation with ¹²⁵I-TGF- β was used to determine the amount of ¹²⁵I-labelled TGF- β bound non-specifically to the surface of cells or plastic dishes. Binding buffer without ¹²⁵I-TGF- β 1 was added to those six-well plates that were used in parallel for cell counting using a Neubauer counting chamber.

To quantify free ligand concentrations, the buffer was removed from the wells cooled on ice and the wells were rinsed twice with 1 ml ice-cold binding buffer. The combined fractions were analysed for free radioactivity in a gamma counter. To quantify bound ligand, the wells were rinsed for three additional times, then 2 ml

solubilization buffer I (25 mM HEPES, 1% (v/v) Triton X-100, 10% (v/v) glycerol, pH 7.5, 1 mg/ml BSA) were added and incubated for 40 min at 4 °C on the platform. The buffer was removed, the wells were rinsed twice and the combined fractions were analysed in a gamma counter. The difference between c.p.m. bound in the presence and absence of excess unlabelled TGF- β was considered to represent specific binding. Saturation analysis was carried out according to Scatchard (1949).

Crosslinking experiments

The crosslinking experiments were performed according to Massagué (1985). Cell monolayers were treated according to the procedure described for the binding studies. After the equilibration step, cells were incubated with 20 pM 125 I-labelled TGF- β 1 in binding buffer at 4 °C on a platform oscillating at 2 Hz for 3.5 h. Next, wells were washed five times with binding buffer lacking BSA and then covered with 2 ml of this buffer, into which 10 ml freshly prepared 27 mM disuccinimidylsuberate (DSS) solution (Pierce, Rockford, USA) in dimethyl sulphoxide were dispensed directly, during swirling. The dishes were left agitating at 4 °C for 15 min. Cells were briefly rinsed with ice-cold detachment buffer (250 mM saccharose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4 supplemented with an inhibitor cocktail, Boehringer Mannheim, Mannheim, Germany) and then scraped off in the presence of 0.5 ml detachment buffer, using a disposable scraper. The wells were rinsed with a second aliquot of detachment buffer. Cells were pelleted by centrifugation at 20 000 g for 2 min at 4 °C in 2-ml microcentrifuge tubes and the supernatant was removed. Cell pellets were resuspended by repeated pipetting with 60 ml solubilization buffer II (125 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.0, 1% (v/v) Triton X-100, supplemented with an inhibitor cocktail) and incubated for 40 min at 4 °C with end-over-end mixing. Insoluble cell debris was removed by centrifugation at 20 000 g at 4 °C for 15 min. The supernatant was mixed with an equal volume of electrophoresis sample buffer and heated at 90 °C for 2 min. Samples were then subjected to SDS-PAGE on 10% polyacrylamide gels. Gels were fixed and dried, followed by autoradiography for up to 30 days using an intensifying screen (Reflection Autoradiography Film and Screen, NEN, Boston, MA, USA).

Immunoblots for T β R-II and betaglycan

Cell monolayers grown to confluency overnight were incubated with 200 nM TGF- β 1 or vehicle for 24 h and 48 h. Medium was then removed from cells on ice. Cell collection and SDS-PAGE were performed according to the crosslinking protocol. Before SDS-PAGE, the protein content in each sample was determined according to Lowry *et al.* (1951) (Lowry, DC Protein Assay kit,

Bio-Rad, Hercules, CA, USA). Equal amounts of protein for each sample were mixed with 1 vol Laemmli sample buffer containing 1% β -mercaptoethanol, heated at 95 °C for 2 min and loaded onto a 10% polyacrylamide gel, followed by electrotransfer to a 0.45-mm nitrocellulose membrane (Bio-Rad). After washing for 5 min in 50 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1% Tween 20 (TBST), the membrane was incubated in blocking buffer (5% BSA in TBST) with gentle agitation overnight at 4 °C. The primary antibody (R&D Systems, Wiesbaden, FRG) was added in a concentration of 25 ng/ml in blocking buffer for 1 h, with agitation, at room temperature, followed by washing for 3 $\times$ 5 min and incubation with the second antibody (donkey anti-goat, conjugated to alkaline phosphatase; Dianova, Hamburg, FRG) in a concentration of 600 ng/ml. After washing, the membrane was equilibrated in substrate buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris-HCl, pH 9.5). Blots were then developed in 20 ml substrate buffer containing 132 ml nitro blue tetrazolium (NBT) stock solution (50 mg/ml NBT in 70% dimethyl-formamide (DMF)) and 66 ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) stock solution (50 mg/ml BCIP in 100% DMF) until the desired degree of staining has been achieved. In an additional experiment, the pellet was analysed for immunoreactivity for both T β R-II and T β R-III. For this purpose, the pellet was solubilized in 2.5% SDS and heated at 95 °C for 2 min before the immunoblotting procedure described above.

Analysis of T β R-II gene expression by reverse transcription (RT)-PCR

For semi-quantitative analysis, T β R-II gene expression was studied by RT-PCR in relation to expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as a coamplified internal standard (Kruse *et al.* 1998). Total RNA from human osteoblast-like cells was isolated using the RNeasy kit (Qiagen, Hilden, FRG). Samples of 2 mg were reverse-transcribed and amplified using sequence-specific oligonucleotide primers for both the T β R-II and the GAPDH gene included in the same reactions. To exclude contamination of genomic DNA as a source for amplified products, each reaction was also carried out without reverse transcriptase. Sequences of the antisense and sense primers were: GAPDH: 5'-GCA ACT GTG AGG AGG GGA GAT TCA G-3', 5'-CCG CAT CTT CTT TTG CGT CGC-3'; T β R-II: 5'-GCC TGC CCC ATA AGA GCT ATT TGG TAG TG-3', 5'-GAA GAA CGA CCT AAC CTG CTG CCT-3'.

PCR was performed on 1/20 of the reverse-transcription reaction using Vent-Polymerase (Biolabs, Schwalbach, FRG) and following the protocols supplied by the manufacturers (each cycle consisted of 40 s of denaturation at 95 °C, 40 s annealing at 56 °C, and 60 s of elongation at 72 °C). Amplification of both GAPDH and

Figure 1 Cell-surface expression of T β R on representative human osteoblast-like cells from adult donors and on cells of the osteosarcoma-derived osteoblastic cell line, MG63. Analysis was performed by crosslinking with DSS after binding of 125 I-TGF- β 1 (-). T β Rs -I (RI), -II (RII) and -III (RIII) (betaglycan) are seen at approximately 80, 100 and 180 kDa respectively. Specificity of binding was demonstrated by inhibition of labelling after preincubation (+) with an excess of unlabelled TGF- β 1. m34, f64 and f59 denote osteoblast populations from male (m) and female (f) donors 34, 64 and 59 years of age respectively.

T β R-II was found to be in the linear range when 30 cycles of amplification were used.

Statistics

Calculation of means and standard deviations presented in Fig. 4 was performed on data derived from four separate experiments with double determination each. Statistical analysis was carried out using ANOVA. Statistical significance was considered for $P < 0.05$.

Results

Cell-surface expression of TGF- β -receptors

The cell-surface expression of T β R on human osteoblast-like cells as revealed by affinity crosslinking studies using 125 I-TGF β is shown in Fig. 1. T β Rs -I, -II and -III (betaglycan) were abundantly present on cultured human osteoblast-like cells as visualized by electrophoretic separation of the receptor-ligand complexes, showing the expected bands migrating at approximately 80, 100 and 280 kDa, respectively, which is in good agreement with data published by other authors (Attisano *et al.* 1994). The specificity of the binding was demonstrated by an inhibition of labelling after preincubation with an excess of unlabelled TGF- β 1. When a total of 20 different human osteoblast populations or transformed osteoblastic cells (MG63) was analysed, virtually the same banding pattern was seen for all cells (representative findings are shown in Fig. 1). In agreement with the data obtained by the binding studies and the immunoblotting described below, preincubation with unlabelled TGF- β 1 also revealed

Figure 2 Specific binding curves of 125 I-TGF- β 1 on human osteoblast-like cells after preincubation with different concentrations (40 pM (Δ) and 200 pM ($\bullet$)) of unlabelled TGF- β 1. $\diamond$, Control values without TGF- β 1 preincubation.

a decrease in all three receptor types in the affinity crosslinking studies (data not shown).

Specific binding of 125 I-TGF- β

To demonstrate the presence of receptors specific for TGF- β on human osteoblast-like cells, binding of 125 I-TGF- β 1 was analysed and specific binding curves were obtained by subtraction of unspecific binding from total binding. Preincubation with 40 pM and 200 pM unlabelled TGF- β 1 for 18 h resulted in a marked and dose-dependent reduction of specific binding of 125 I-TGF- β (Fig. 2). Different preincubation times with unlabelled TGF- β 1 (200 pM) revealed a time-dependent significant reduction of specific binding of 125 I-TGF- β . Binding was greater after 30 min than after 2 h and 4 h preincubation, and was least after 18 h preincubation (Fig. 3a). Further analysis of the binding data showed that this represented significant reductions (53, 84, 90 and 95% compared with control) in the presence of cell-surface receptors able to bind TGF- β 1 after ligand preincubation for 0.5, 2, 4 and 18 h, respectively, while the receptor affinity did not change significantly under the different preincubation conditions (Fig. 4). No recovery of receptor binding capacity was observed for up to 18 h in the presence of the ligand (Fig. 3a). Also, there was no evidence for receptor recovery for up to 24 h after TGF- β 1 had been removed (Fig. 3b).

Control experiments using 125 I-TGF- β instead of unlabelled TGF- β in the first incubation also showed a

Figure 3 (a) Number of T β R_s per cell and their significant decrease after preincubation with unlabelled TGF- β 1 for different durations (0, 0.5, 2, 4 and 18 h). (b) Number of T β R_s per cell after an 18-h preincubation with TGF- β 1. Different time points after the removal of TGF- β 1 are shown (0, 4, 12 and 24 h). Values in both (a) and (b) are expressed as the ratio of receptor number (mean $\pm$ s.d. of four separate experiments) with and without TGF- β preincubation and normalized to the value observed without TGF- β 1 preincubation (C, control).

reduction of specific ligand binding and thus indicated that no residual occupancy of cellular receptors was present.

Immunoblotting analyses

To analyse the influence of preincubation with TGF- β on the cell-surface expression of individual T β R proteins,

Figure 4 Scatchard analysis of the binding data obtained by the specific binding of 125 I-TGF- β 1 on human osteoblast-like cells after preincubation with unlabelled TGF- β 1 for 2 ($\blacktriangle$), 4 ($\square$) and 18 ($\bullet$) h. $\diamond$, Control values without TGF- β 1 preincubation. There is decreased specific binding capacity (derived from the intercept on the abscissa) with TGF- β 1 preincubation, without change in affinity (slope).

immunoblotting analyses were carried out under denaturing conditions, which completely disrupt any ligand-receptor binding. Because T β R-II can bind TGF- β independently of T β R-I, and binding to T β R-II is the first step in TGF- β signalling (Heldin *et al.* 1997), immunoblotting was performed with antibodies against T β R-II. The semi-quantitative analysis showed an almost complete disappearance of immunoreactivity for T β R-II after a 24-h and 48-h preincubation with 200 pM TGF- β 1 (Fig. 5a; data shown are for 24 h), under both reducing and non-reducing conditions. In addition, immunoblotting with specific antibodies against betaglycan was also performed, because betaglycan (T β R-III) seems to have a role in presenting TGF- β to T β R-II (López-Casillas *et al.* 1994). As shown in Fig. 5a, the T β R-III core protein (López-Casillas *et al.* 1994) was almost undetectable by specific antibodies after preincubation (for 24 or 48 h; data shown are for 48 h) with TGF- β 1 (40 pM). Analysis of the solubilized pellet failed to show any immunoreactivity for either T β R-II or T β R-III, and gave the same results with or without TGF- β preincubation (Fig. 5b). It therefore appears unlikely that the receptors had been moved to an insoluble membrane compartment in the course of the experiments.

Expression of T β R-II mRNA

To investigate the influence of a 24-h preincubation with TGF- β 1 on the expression of T β R-II mRNA,

Figure 5 Immunoblotting analyses of TβRs-II and -III. (a) Specific bands at a molecular weight of approximately 80 and 110 kDa (core protein), respectively, on human osteoblast-like cells with (+) or without (-) TGF-β1 preincubation for 24 h (TβR-II) and 48 h (TβR-III). (b) Analyses of the solubilized pellet with (+) and without (-) TGF-β1 preincubation, and control (C).

semi-quantitative RT-PCR was performed using GAPDH mRNA levels as internal standard. As shown in Fig. 6, there was no significant effect of TGF-β1 on the expression of TβR-II mRNA. The marked changes in receptor binding profiles and receptor immunoreactivity observed after incubation with TGF-β1 therefore apparently were not paralleled by alterations in the TβR-II transcript levels.

Discussion

It is generally accepted that TGF-β may have a pivotal role in co-ordinating bone deposition and resorption (Bonewald & Dallas 1994, Centrella *et al.* 1994) and may therefore represent a candidate factor for coupling both processes. In addition, there is increasing evidence that TGF-β exerts a critical local function in the skeleton with regard to augmenting or maintaining bone mass and its structural integrity. In this respect, a net bone loss or bone gain has been shown to be selectively induced by over-expression of TGF-β or inhibition of TβR function, respectively (Erlebacher & Derynck 1996, Filvaroff *et al.* 1997). The most pronounced effect of TGF-β is the

stimulation of the synthesis of bone matrix proteins, including collagen I, proteoglycans and fibronectin, and its potential to influence the functional properties of the collagenous bone matrix (Takeuchi *et al.* 1996, Seitzer *et al.* 1995). In contrast, there is convincing evidence that TGF-β inhibits the final mineralization process during bone maturation (Harris *et al.* 1994, Takeuchi *et al.* 1996), which provides further support for a tight regulation of its action during tissue formation and remodelling of bone. To date, little is known about how the TGF-β-responsiveness of bone-forming cells, which possess three types of TβRs, might be regulated in the human skeletal system. Some, but not all, studies using rodent or transformed mesenchymal cells have reported a downregulation of receptors for TGF-β by the ligand itself (Massagué & Like 1985, Wakefield *et al.* 1987, Centrella *et al.* 1996). The present study on adult human osteoblast-like cells provides additional evidence that TGF-β1 reduces the number of specific TβRs per cell exposed on the cell surface, in a dose- and time-dependent manner. Any residual occupancy of cellular receptors resulting from the preincubation was excluded by control experiments using ¹²⁵I-labelled TGF-β1 instead of unlabelled TGF-β1 in the first incubation, which also resulted in a decreased total binding of ¹²⁵I-TGF-β1 after the second incubation. In the case of residual binding from the first incubation, one would expect identical or higher labelling values (Massagué & Like 1985). Furthermore, there was a dose-dependent effect of ligand binding even after exposure times longer and with ligand concentrations greater than necessary for a complete saturation of all receptors. Finally, the immunoblotting experiments with antibodies against TβRs -II and -III were performed under conditions that completely disrupt any receptor-ligand binding, and they also revealed a drastically reduced amount of receptor protein after TGF-β preincubation.

Figure 6 TβR-II (R II) mRNA analysed by RT-PCR. There was no change in the RII:GAPDH mRNA ratio after a 24-h incubation with TGF-β1 (+) compared with control (-). M, Marker.

The reduction of the cell-surface binding capacity was about 84% and 90% after 2 and 4 h of preincubation with 5 ng/ml TGF- β , respectively, and approached 100% after 18 h. In addition, the effect of preincubation with TGF- β showed a clear dose-dependency. Earlier studies using fetal rat fibroblasts or rodent cell lines failed to show a marked receptor downregulation after a 2-h treatment with unlabelled TGF- β (Massagué & Like 1985, Wakefield *et al.* 1987). In contrast, our data are mostly in accordance with the results of a recent study by Centrella *et al.* (1996) on fetal rat calvarial cells. However, in that study a return of the full cell-surface binding capacity after 24–48 h at lower concentrations of TGF- β was reported, and a persistent downregulation was observed after exposure to 400 pM TGF- β . Our data obtained by complete binding studies would suggest that, in non-fetal human osteoblast-like cells, there seems to be a near total (>90%) ligand-induced downregulation of T β Rs -II and -III at a concentration of 200 pM and greater after an exposure time of 4 h and longer, without any evidence for receptor recovery within the first 24 h as shown by immunoblotting. Furthermore, binding studies revealed that no receptor recovery was present for up to 24 h after the exposure to TGF- β had been stopped.

The ligand-induced downregulation of receptors for TGF- β could be biologically significant with regard to the changing role of TGF- β during osteoblastic differentiation. The abundance of TGF- β in bone tissue may exceed the amount needed for osteoblast function during certain stages of skeletal organogenesis. As TGF- β strongly inhibits the expression of ALP and osteocalcin and the formation of mineralized bone nodules (Wrana *et al.* 1988, Noda 1989, Harris *et al.* 1994), a tight regulation of the actions of TGF- β is important in later stages of bone tissue formation. A differentiation-associated downregulation of T β Rs mediated by interaction between murine osteoblasts and matrix collagen has been reported, and osteoblastic differentiation of multipotential mesenchymal cells induced by retinoic acid was shown to reduce the cell surface binding of TGF- β (Gazit *et al.* 1993, Takeuchi *et al.* 1996). In contrast, an upregulation of type I receptors for bone morphogenetic proteins, which also belong to the TGF- β superfamily but, in contrast to TGF- β , have a stimulatory effect on mineralization, has been shown to occur during bone formation (Ishidou *et al.* 1995). The data obtained in our study on human osteoblasts suggest that TGF- β 1, which is present in high concentrations in the osteoblastic microenvironment and released from accumulated matrix during bone resorption, is able to reduce the number of its own cell-surface receptors. In contrast, no significant effect on receptor affinity was observed. Thus one might speculate that, during the late steps of osteosynthesis such as the mineralization phase, the inhibitory effect of high concentrations of TGF- β 1 during bone remodelling

could be suppressed, as lower levels of T β Rs would enable mineralization in regions where the organic bone matrix is already sufficiently formed. The concentrations of active TGF- β 1 applied in our *in vitro* incubation experiments are unlikely to be supraphysiological, and are even lower than those reported recently for human bone tissue (Nicolas *et al.* 1994, Pfeilschifter *et al.* 1998). However, it cannot completely be ruled out that an activation of latent TGF- β 1 stored in the matrix occurred during the extraction procedure, which might have resulted in higher concentrations of detectable TGF- β 1 when only active TGF- β 1 was measured.

The mechanism by which TGF- β -binding receptors are decreased appears not to be based on transcriptional events, because the amount of T β R-II mRNA was not significantly decreased by TGF- β in our study. Rather, on the basis of results of the semi-quantitative RT-PCR, one may speculate that the decrease is due to post-transcriptional effects, for example changes in the intracellular trafficking of receptor proteins. Both endocytotic and degradational downregulation of receptors have been described as general mechanisms, depending on whether an increased internalization or degradation is induced by the ligand (Zigmond *et al.* 1982, Wiley *et al.* 1985). Similar mechanisms have been suggested regarding the collagen-induced downregulation of T β Rs, which likewise was not paralleled by a reduction in the abundance of the transcript for T β R-II (Takeuchi *et al.* 1996). An additional possibility is that intracellular protein phosphorylation influences the binding capacity of the cell-surface receptors. Treatment with protein kinase inhibitors has recently been shown to alter TGF- β binding (Centrella *et al.* 1996). However, while an altered accessibility of labelled ¹²⁵I-TGF- β 1 to its receptors cannot be ruled out, our results obtained by immunoblotting of T β Rs -II and -III and performed after cell lysis at least also suggest a reduced amount of T β R-II and T β R-III protein induced by preincubation with TGF- β 1, without any evidence for an altered receptor affinity. Further investigation is required on the precise mechanisms of ligand-induced downregulation of receptors for TGF- β on human osteoblast-like cells.

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