TGF-β₁ As Possible Link between Loss of Bone Mineral Density and Chronic Inflammation

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Abstract

Background: The TGF family plays a key role in bone homeostasis. Systemic or topical application of proteins of this family apparently positively affects bone healing in vivo. However, patients with chronic inflammation, having increased TGF-β₁ serum-levels, often show reduced bone mineral content and disturbed bone healing. Therefore, we wanted to identify intracellular mechanisms induced by chronic presence of TGF-β₁ and their possible role in bone homeostasis in primary human osteoblasts.

Methodology/Principal Findings: Osteoblasts were isolated from femur heads of patients undergoing total hip replacement. Adenoviral reporter assays showed that in primary human osteoblasts TGF-β₁ mediates its signal via Smad2/3 and not Smad1/5/8. It induces proliferation as an intermediate response but decreases AP-activity and inorganic matrix production as a late response. In addition, expression levels of osteoblastic markers were strongly regulated (AP ↓; Osteocalcin ↑; Osteopontin ↑; MGP ↓; BMP 2 ↓; BSP2 ↓; OSF2 ↓; Osteoproteregenin ↓; RANKL ↑) towards an osteoclast recruiting phenotype. All effects were blocked by inhibition of Smad2/3 signaling with the Alk5-Inhibitor (SB431542). Interestingly, a rescue experiment showed that reduced AP-activities did not recover to base line levels, even 8 days after stopping the TGF-β₁ application.

Conclusions/Significance: In spite of the initial positive effects on cell proliferation, it is questionable if continuous Smad2/3 phosphorylation is beneficial for bone healing, because decreased AP-activity and BMP2 levels indicate a loss of function of the osteoblasts. Thus, inhibition of Smad2/3 phosphorylation might positively influence functional activity of osteoblasts in patients with chronically elevated TGF-β₁ levels and thus, could lead to an improved bone healing in vivo.

Introduction

Throughout life, bone undergoes continuous remodeling by a coordinated process of bone formation and bone resorption. Bone is formed by osteoblasts, which are of mesenchymal origin, and is resorbed by osteoclasts, derived from the hematopoietic system. Both actions are closely linked to maintain constant bone mass in the adult skeleton. Deregulation of this balance underlies the pathological loss of bone mass seen with delayed bone healing after fracture, osteoporosis and other metabolic bone diseases. Despite of their importance for our understanding of normal bone metabolism and the pathogenesis of metabolic bone diseases, the molecular mechanisms that govern the coordination of these processes are largely unknown.

Bone morphogenic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, are able to promote osteogenesis, chondrogenesis and adipogenesis, whereas they inhibit myogenesis of mesenchymal progenitor cells [1]. However, as TGF-β is by far the most abundant cytokine in bone, by its mere abundance (200 μg/kg), it must be considered as a central player in bone turnover [2]. Both osteoblasts and osteoclasts secrete all three TGF-β iso-forms (TGF-β₁, -β₂ and -β₃), which are present in their latent form within bone matrix [3,4]. During bone turnover, acidification of the resorption lacuna by osteoclasts is thought to activate TGF-β [5], which should then stimulate the formation of bone [6]. Systemic or topical application of proteins of this family apparently positively affects bone healing in vivo. However, TGF-β is also strongly expressed during various inflammation reactions. Patients with liver fibrosis or cirrhosis often show elevated TGF-β levels over a long period [7,8]. Similar results are seen in cardiac fibrosis, chronic renal failure or fibrosis of other tissues [9,10,11]. Thus, we propose that chronically increased serum levels of TGF-β₁, observed in many systemic diseases might be a potential inducer for associated loss of bone density, as seen in hepatic or renal osteodystrophy.

Members of the TGF-β superfamily transduce their signals through two types of serine/threonine kinase receptors, termed...
type I and type II [12,13]. The type II receptors are constitutively active kinases which phosphorylate type I receptors upon ligand binding. Seven type I receptors termed activin receptor-like kinase (Alk)-1 through -7 have been identified in mammals. BMPs, activins and TGF-β bind to different type I receptors, depending on the cell type. BMPs preferably bind to Alk2, -3 and -6, whereas activins and TGF-β bind to Alk-4 and Alk-5, respectively. Upon activation by type II receptors, Alk activate (phosphorylate) transcription factors, so-called Smads, in the cytoplasm. Eight different Smads have been identified in mammals. They are classified into three groups: receptor-regulated Smads (R-Smads/Smad1, -2, -3, -5 and -8), inhibitory Smads (I-Smads/Smad6 and -7) and the common-partner Smad (Co-Smad/Smad4). Alk1, -2, -3 and -6 activate Smad1/5/8 while Alk4, -5 and -7 activate Smad2/3 [14]. Upon activation Smad1/5/8 and Smad2/3 form complexes with Smad4, which allows them to translocate into the nucleus for regulation of target gene expression [15]. The signaling cascade of Smad2/3 can be inhibited by the chemical inhibitor SB431542, which has been shown to inhibit Alk4, -5, and -7 kinase activity specifically, but not Alk2, -3, and -6 kinase activity [16].

TGF-β1 is a multifunctional signaling protein that initiates a wide variety of responses in many different cell types. Thus, TGF-β1 is involved in embryogenesis, differentiation, wound healing, extracellular matrix (ECM) production and cell-cell adhesion [17,18,19]. Osteoblasts express a large variety of high affinity TGF-β receptors and therefore, TGF-β is thought to regulate many osteoblastic functions including expression of ECM genes, e.g. collagen and fibronectin, their integrin receptors and even the stabilization of integrin subunits [4,20,21,22]. From the TGF-β superfamily, TGF-β1 showed the strongest chemotactic effect towards human osteoblasts, thus application of this cytokine in a dog model was able to enhance mechanical fixation, bone ingrowth and gap bone formation with unloaded implants surrounded by a gap. This effect was pronounced only with low concentrations of TGF-β1 but not with higher concentrations [23]. In vitro effects of TGF-β vary substantially depending upon the cell system. Neonatal and fetal organ cultures have generally indicated that TGF-β inhibits osteoclast differentiation from bone marrow monocytes, yet stimulates bone resorption by differentiated osteoclasts [24,25]. From these results it is hard to draw conclusions for the normal role of TGF-β in bone development. Besides its complex and variable effects on bone cell populations in vitro and in vivo, a given experimental result with TGF-β may be potentially relevant to many different aspects of skeletal morphogenesis, including the generation of bone shape, bone growth, or bone remodeling. Thus, aim of this study was to investigate the long term effects of TGF-β1 on primary human osteoblasts in terms of signaling, proliferation, alkaline phosphatase (AP) activity, osteogenic marker gene expression and mineralized matrix formation.

Results

TGF-β1 increases proliferation but decreases AP activity in primary human osteoblasts

Primary human osteoblasts were treated with different conc. (0, 1, 2.5, 5, 10 ng/ml) of human recombinant active (hra) TGF-β1. After 8 days AP-activity was measured and adherent cells were fixed for SRB staining of surface proteins. SRB staining confirmed the microscopic observation that TGF-β1 induces proliferation in primary human osteoblasts in a dose dependent manner (Figure 1A). At the same time AP-activity was significantly reduced, dose-dependently (Figure 1B).

TGF-β1 mediates its signal via Smad2/3 in primary human osteoblasts

Primary human osteoblasts were infected with adenoviral reporter constructs (Ad5-CAGA9-MLP-Luc or Ad5-BRE-Luc) as described in materials and methods. After infection, cells were stimulated with 5 ng/ml hra TGF-β1. Cell lysates were taken after 24 h (Ad5-CAGA9-MLP-Luc) or 48 h (Ad5-BRE-Luc) and luciferase activity was measured. TGF-β1 increased only Smad3 regulated luciferase signal (Ad5-CAGA9-MLP-Luc) by 9.3-fold. Induction was completely inhibited by the Alk5 inhibitor SB431542, in a dose-dependent manner (Figure 2A). In contrast to BMP2 (1.97±0.18 fold; p<0.001) or BMP7 (2.24±0.21 fold; p<0.001), hra TGF-β1 was not able to induce Smad1 dependent luciferase expression.

Increased proliferation and decreased AP-activity by TGF-β1 is dependent on Smad2/3 signaling and can be reversed by Alk5 inhibitor SB431542

Primary human osteoblasts were stimulated with 5 ng/ml hra TGF-β1 with or without different conc. of Alk5 inhibitor SB431542 (5, 10, 20, 40 nM) for 8 days. Cells were also infected with adenoviral constructs expressing constitutive active Alk5 (Ad5-caAlk5) for 4 days. In this setting, the constitutive active Alk5 ensures activation of the Smad2/3 pathway without additional stimulation with TGF-β1. AP-activity was measured and adherent cells were fixed for SRB staining of surface proteins. SRB showed that TGF-β1-dependent induction of proliferation in primary human osteoblasts can be inhibited by the Alk5 inhibitor SB431542 in a dose-dependent manner. Infection of cells with caAlk5 also led to increased SRB staining (Figure 2B). Interestingly, SB431542 not only inhibited the reduction of AP-activity, but even seemed to increase AP-activity dose-dependently (Figure 2C).

Reversal of TGF-β1-dependent effects on AP-activity by SB431542 is time-dependent

Primary human osteoblasts were stimulated with 5 ng/ml hra TGF-β1 with or without 20 nM Alk5 inhibitor SB431542. After 4, 8 and 12 days of continuous stimulation with TGF-β1 AP activity was measured for half of the cells. The other half of the cells was washed twice with DPBS and culturing was continued for 8 days with basic culture medium. After the additional 8 days AP activity was measured again. With increasing time AP-activity was further reduced by TGF-β1 treatment. For all time-points the Alk5 inhibitor SB431542 was able to block the TGF-β1-dependent decrease of AP activity. With increasing time of TGF-β1 pre-treatment, the so-called “rescue effect”, observed after the additional 8 days in basic culture medium, was reduced (Figure 3A-C).

TGF-β1 inhibits formation of mineralized matrix in primary human osteoblasts

During osteogenic differentiation primary human osteoblasts were stimulated with 5 ng/ml hra TGF-β1 in the presence or absence of 20 nM Alk5 inhibitor SB431542. After 20 days mineralized ECM was stained with Alizarin Red or von Kossa. Von Kossa staining showed that constant treatment of primary human osteoblasts with TGF-β1 inhibited production of mineralized matrix (Figure 4A). Alizarin Red staining revealed that formation of mineralized matrix was reduced to 49.1±8.1% of control cells. This effect was partially reversed (81.9±8.0%) by the Alk5 inhibitor SB431542 (Figure 4B).
**TGF-β₁ increases secretion of RANKL into the culture supernatant**

RANKL levels in culture supernatants of human osteoblasts (N = 3, n = 4), stimulated for 8 days with 5 ng/ml hra TGF-β₁ with or without 20 nM Alk5 inhibitor SB431542, were measured by ELISA. TGF-β₁ increased RANKL secretion by 2.22 ± 0.12 fold (p < 0.001). Stimulation with SB431542 alone did not significantly alter RANKL levels in the culture supernatant (0.04 ± 0.07 fold of control). However, SB431542 was able to significantly reduce the TGF-β₁-dependant increase in RANKL secretion by 66.7 ± 2.7% (p < 0.001).

**TGF-β₁ regulates expression of osteoblast marker genes**

Primary human osteoblasts were stimulated with 5 ng/ml hra TGF-β₁ with or without 20 nM Alk5 inhibitor SB431542. At the same time cells were infected with Ad5-caAlk5 virus particles. After 8 days we isolated mRNA for expression analysis. RT-PCRs were performed for AP, collagen1 (Col1), osteocalcin (OC), osteopontin (OP), osteonectin (ON), BMP2, bone sialoprotein (BSP) 2, matrix gla protein (MGP), osteoblast specific factor (OSF) 2 and osteoprotegerin (OPG). GAPDH was used as housekeeping gene (Figure 5). Densitometric analysis (Table 1) showed that TGF-β₁ treatment reduced AP mRNA levels compared to

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**Figure 1. TGF-β₁ increases proliferation but decreases AP activity in cultured osteoblasts.** Primary human osteoblasts were treated for 8 days with different conc. (0, 1, 2.5, 5, 10 ng/ml) of hra TGF-β₁. (A) SRB staining of surface proteins showed that TGF-β₁ dose-dependently increased proliferation of osteoblasts. (B) In contrary, AP activity, normalized to relative cell numbers, was decreased by TGF-β₁ in a dose-dependent manner. Results are expressed as mean ± standard deviation (N = 4, n = 4). **p < 0.01, ***p < 0.001 in comparison to untreated cells. doi:10.1371/journal.pone.0014073.g001

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**Figure 2. TGF-β₁ mediated changes in primary human osteoblasts are Smad2/3-dependent.** (A) Primary human osteoblasts (N = 4, n = 3) infected with Ad5-CAGA-Luc reporter constructs (Smad2/4 reporter) were stimulated with 5 ng/ml hra TGF-β₁ in the presence or absence of the Alk5 inhibitor SB431542 (5, 10, 20 and 40 nM). After 24 h luciferase activity was measured in cell lysates and normalized to total protein contents. (B/ C) Primary human osteoblasts (N = 4, n = 4) were stimulated with 5 ng/ml hra TGF-β₁ with or without the SB431542 (5, 10, 20, 40 nM) for 8 days or infected with Ad5-caAlk5 for 4 days. (B) Both TGF-β₁ stimulation and caAlk5 infection increased proliferation (SRB staining) in primary human osteoblasts, which was inhibited dose-dependently by SB431542. (C) In the same line, AP activity, normalized to relative cell numbers, was decreased by TGF-β₁ stimulation and infection with caAlk5. The decrease in AP-activity was blocked by SB431542 in a dose-dependent manner. Results are expressed as mean ± standard deviation. **p < 0.01, ***p < 0.001 in comparison to only TGF-β₁ treated cells. doi:10.1371/journal.pone.0014073.g002
untreated cells, confirming results from AP activity measurement. In addition, expression levels of BMP2 and OSF2, involved in osteoblasts recruitment and adhesion, were reduced by TGF-β1 treatment and Ad5-caAlk5 infection. Coll1 and ON mRNA levels were not significantly altered. Genes involved in matrix mineralization, e.g. OC, BSP and MGP, were significantly down-

Figure 3. TGF-β1 dependent effects on AP activity by SB431542 are depending on time. Primary human osteoblasts stimulated with 5 ng/ml hra TGF-β1 in the presence or absence of 20 nM SB431542. After (A) 4, (B) 8 and (C) 12 days of continuous stimulation with TGF-β1 AP activity was measured and normalized to SRB staining for half of the cells (empty bars). The remaining cells were washed twice with DPBS and continued culturing for 8 days with basic culture medium. After the additional 8 days AP activity was measured in the same way (filled bars). Overall, with increasing time AP-activity was more and more reduced in the presence of TGF-β1. Supplementation with SB431542 was able to block the TGF-β1-dependent decrease of AP activity at all time-points. However, with increasing time of TGF-β1 pre-treatment, the so-called “rescue effect”, observed after the additional 8 days in basic culture medium, was reduced. Results are expressed as mean ± standard deviation (N = 4, n = 4). *p < 0.05, **p < 0.01, ***p < 0.001 versus the corresponding untreated cells.

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Figure 4. TGF-β1 inhibits formation of mineralized matrix in primary human osteoblasts. Primary human osteoblasts cultured in differentiation medium with 5 ng/ml hr TGF-β1 with or without 20 nM SB431542 for 20 days. (A) Representative picture for von Kossa staining for mineralized matrix. (B) Quantitative staining of produced mineralized matrix with Alizarin Red. Continuous stimulation of cells with TGF-β1 inhibited formation of mineralized matrix. We could prevent this effect by addition of SB431542. Results are expressed as mean ± standard deviation (N = 5, n = 6). ***p < 0.001 in comparison to control conditions.

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regulated by both treatments. In contrast, mRNA levels for OP, which favors osteoblast binding is increased by stimulation with TGF-$\beta_1$ and over-expression of caAlk5. In the same line, mRNA levels of OPG, inhibiting osteoclast differentiation and activity, were reduced by both treatments. All observed effects were reversed by co-incubation with the Alk5 inhibitor SB431542.

**TGF-$\beta_1$ reduces BMP2 and BMP7 signaling in human osteoblasts**

Primary human osteoblasts were infected with Ad5-BRE-Luc adenoviral particles (Smad1/4 reporter construct) and stimulated with 5 ng/ml hra TGF-$\beta_1$, or/and 50 ng/ml hra BMP2 or -7. Cell lysates were taken after 48 h and luciferase activity was measured. In contrast to BMP2 (2.0±0.2 fold) and BMP7 (2.2±0.2 fold), TGF-$\beta_1$ failed to induce Smad1 regulated luciferase signal. Interestingly, BMP2 and -7 induced luciferase signal was blocked by co-incubation with TGF-$\beta_1$ (Figure 6).

**Table 1. Densitometric analysis of RT-PCR.**

| Gene | Control | 5 ng/ml TGF-$\beta_1$ | 5 ng/ml TGF-$\beta_1$, 20 nM SB431542 | Ad5-caAlk5 |
|------|---------|----------------------|---------------------------------|------------|
| AP   | 1.00±0.11 | 0.39±0.05 | 0.94±0.10 | 0.62±0.08 |
| BMP2 | 1.00±0.16 | 0.57±0.21 | 1.11±0.13 | 0.37±0.04 |
| OSF2 | 1.00±0.14 | 0.58±0.13 | 0.85±0.13 | 0.76±0.11 |
| Coll1| 1.00±0.12 | 0.86±0.12 | 0.91±0.12 | 0.88±0.08 |
| OC   | 1.00±0.16 | 0.40±0.06 | 1.02±0.17 | 0.51±0.11 |
| ON   | 1.00±0.19 | 1.00±0.19 | 1.27±0.24 | 1.16±0.22 |
| BSP2 | 1.00±0.14 | 0.22±0.03 | 1.61±0.25 | 0.47±0.09 |
| MGP  | 1.00±0.10 | 0.83±0.10 | 1.38±0.19 | 0.65±0.07 |
| OP   | 1.00±0.08 | 1.34±0.28 | 0.72±0.09 | 1.17±0.08 |
| OPG  | 1.00±0.08 | 0.51±0.04 | 1.08±0.11 | 0.26±0.05 |

N = 4, n=5.

**Discussion**

TGF-$\beta$ is secreted by bone cells and therefore bone represents one of the biggest reservoirs for all three TGF-$\beta$ iso-forms (TGF-$\beta_1$, -$\beta_2$, and -$\beta_3$) of the human body. However, in bone matrix they are present in their latent form [4]. During bone resorption by osteoclasts, pH is decreased and this acidification is thought to activate the TGF-$\beta$ reservoir which should then stimulate the formation of bone [5]. However, patients with chronic inflammation, often have constantly increased active TGF-$\beta_1$ levels due to macrophage activation at the inflammation site. The distribution of this growth factor throughout the body via the bloodstream might influence other organs.

As osteoblasts contain a large variety of high affinity TGF-$\beta_1$ receptors, TGF-$\beta_1$ is thought to regulate many osteoblastic functions including expression of ECM genes, e.g. collagen and fibronectin, and the corresponding integrin receptors [4,20,21,22]. Furthermore, it has been suggested that the down-regulation of cytokines and hormones, such as IGF-1, IL-11 and growth hormone, but also TGF-$\beta_1$, is correlated with age related bone loss [26,27,28]. This hypothesis was supported by in vitro studies showing that TGF-$\beta$ increases synthesis of type I collagen and non-collagenous bone proteins by cultured osteoblastic cells [2,17,29]. However, in our set-up of continuous TGF-$\beta_1$ stimulation type I collagen mRNA did not alter. Moreover, matrix mineralization was blocked completely. In contrary, in vitro TGF-$\beta_1$ knockout mice display a decrease of about 30% in tibia length and a reduction in bone mineral content [30], indicating the need for TGF-$\beta_1$ in bone formation. Furthermore, local injection of TGF-$\beta_1$ under the periosteum stimulated cartilage and bone formation [6,31] while systemic injection of TGF-$\beta_2$ leads to a general increase in osteoblastic activity [32]. In contrast, transgenic mice with osteoblasts specific (ostecalcin promoter) over-expression of TGF-$\beta_2$ show a dramatic, age-dependent loss of bone mass similar to that seen in osteoporosis and hyperparathyroidism [33]. Furthermore, transgenic mice over-expressing osteoblast specific cytoplasmically truncated type II TGF-$\beta$ receptor show decreased bone remodeling and increased trabecular bone mass with tougher femurs and stiffer and stronger vertebral bodies [34]. Similar results were observed when TGF-$\beta$ type I receptor kinase was pharmacologically inhibited by SD-208. These mice showed increased bone mass and multiple aspects of bone quality as trabecular bone architecture and macro-mechanical behavior of vertebral bone [35]. Due to these, often
contradictory, we investigated the effects of TGF-β1 on primary human osteoblasts regarding proliferation, AP-activity, formation of mineralized matrix and osteogenic marker gene expression during a culture period of 20 days, thereby focusing on the underlying signaling cascades and mechanisms. Osteoblasts were isolated from femoral heads of patients undergoing total hip replacement. The average age of the patients was 75.25 years, which resembles the age of patients having an increased incidence of fractures because of loss of bone mineral density due to systemic disorders. In order to perform the experiments with a homogeneous population of primary osteoblasts we cultured and expanded our cells until passage 3 or 4 (up to 4 month) under standard culture conditions. Thus, the addition of TGF-β1 resembles the conditions observed in patients with chronic inflammation. During the first days TGF-β1 strongly induced proliferation of primary human osteoblasts. One possible mechanism might be an interaction between TGF-β1 signaling and Ras activation with subsequent stimulation of MAPKinasas ERK, p38 and JNK [36]. This is supported by the in vivo findings showing that exogenously administered recombinant TGF-β1 was able to increase bone formation and to promote fracture healing [37]. TGF-β1, as well as PDGF-BB, showed the strongest chemotactic effects towards human osteoblasts and thus was able to enhance mechanical fixation, bone ingrowth and gap bone formation in a dog model with unloaded implants surrounded by a gap. Noteworthy, this effect was only present with low concentrations of TGF-β1 but did not appear any more with higher concentrations of this cytokine [23]. This is supported by our results showing that, although the observed increase in proliferation was more pronounced with increasing TGF-β1 concentrations, AP activity and formation of mineralized matrix was dose-dependently reduced. This effect was not only dose- but also time-dependent. Thus, a “rescue” experiment, documenting AP activity, showed that cells could not recover completely from TGF-β1 stimulation for more than 8 days. Thus, in patients with chronic inflammation, having significantly increased levels of circulating TGF-β1 for up to several years [7,8], this might be a key regulator for reduced bone mineralization. Moreover, mRNA analysis revealed that gene expression of treated osteoblasts was shifted from osteoblasts maturation to osteoclast recruitment after only 8 days. For example osteopontin, favoring osteoclast binding, was induced by TGF-β1 in our cell system. Furthermore, we could show a significant increase in RANKL secretion when TGF-β1 is used as stimulus. At the same time OPG, an inhibitor for osteoclast differentiation and activity, was down-regulated in primary human osteoblasts. In contrast, in stromal cells TGF-β1 is reported to stimulate OPG and other factors that indirectly inhibit osteoclastogenesis [38]. Thus, it is conceivable that endogenous TGF-β1 is in the initial step essential for osteoclastogenesis induced by a combination of receptor activator of NF-κB ligand (RANKL) and M-CSF [39]. Interestingly, TGF-β1 did not significantly alter Coll expression in our experiments, but all genes involved in matrix mineralization, explaining the results observed by von Kossa and Alizarin Red staining for mineralized matrix. The observed effects of TGF-β1 could be inhibited by SB431542, which has been shown to inhibit ALK-4/5/7 kinase activity specifically, but not ALK2/3/6 kinase activity responsible for BMP signaling via Smad1/5/8 [16]. Therefore, we propose that the functional loss of osteoblast activity by TGF-β1 treatment is mediated via Smad2/3 signaling. This is supported by our findings that over-expression of constitutive active Alk5 induced similar effects as TGF-β1 in primary human osteoblasts. As expression of cAlk5 is independently of receptor binding activating Smad2/3 phoshorylation, our data clearly suggest that the observed effects are mediated by Smad2/3 signaling. Analysis of possible signaling cascades by adenoviral reporter assays revealed that TGF-β1 only activated Smad2/3/4/5/8 signaling but not Smad1/5/8 signaling as BMP2 or -7. Interestingly, BMP-mediated Smad1/5/8 signaling was reduced by TGF-β1 co-incubation, which might be an explanation for the observed functional loss of the osteoblasts. One possible mechanism for this inhibition might be increased expression of Smad6, as observed in TGF-β1 stimulated C2C12 cells [40].

As bone repair is one of the most important and urgent subjects for our over-aging society, members of the TGF-β superfamily, e.g. TGF-β1 and BMPs, are expected to be applied to the treatment of various orthopedic diseases including bone fracture and spinal fusion [18,41]. Cell adhesion is the first crucial step for osseointegration as it specifies the fate of the cell. Furthermore, proliferation, differentiation, ECM production and organization as well as apoptosis are influenced by the events of cell-substrate adhesion [42,43,44]. However, as described before, effects of TGF-β vary substantially depending upon the cell system. While in neonatal and fetal organ culture TGF-β generally inhibited osteoclast differentiation from bone marrow monocytes, it stimulated bone resorption by differentiated osteoclasts [24,25]. The normal role of TGF-β in bone development has been hard to infer from these studies. Besides its complex and variable effects on bone cell populations in vitro and in vivo, the exogenous application of TGF-β does not mimic its route of production within bone, where it is produced and stored largely as a bone matrix-bound latent complex that may be unable to induce cellular responses unless first released from mineralized bone during osteoclastic bone resorption [45]. Thus, bone cell populations in vitro and in vivo, the exogenous application of TGF-β does not mimic its route of production within bone, where it is produced and stored largely as a bone matrix-bound latent complex that may be unable to induce cellular responses unless first released from mineralized bone during osteoclastic bone resorption [45]. TGF-β is strongly expressed during various inflammation reactions. Patients with liver fibrosis or cirrhosis often show elevated TGF-β levels [7,8]. Similar results are seen in cardiac fibrosis, chronic renal failure or fibrosis of other tissues [9,10,11]. Thus, we propose that chronically increased serum levels of TGF-β1 observed in many systemic diseases might be a potential inducer for associated loss of bone density, as seen in hepatic or renal osteodystrophy. Thus, understanding the underlying mechanisms is mandatory to provide future possible therapeutic concepts for delayed fracture healing and metabolic...
Materials and Methods

Human recombinant active TGF-β1 (Peprotech, London, UK); Cell Culture Medium and supplements (PAA, Colbe, Germany); Chemicals were obtained from Sigma (Munich, Germany). Isolation and culture of primary human osteoblasts

Ethics Statement

Osteoblasts were isolated from femur heads of patients undergoing total hip replacement, in accordance to the ethical vote of the MRI (“Ethikkommission der Fakultät für Medizin der Technischen Universität München”, http://www.ek.med.tum.de, Project Number 2413, TU Munich, Germany) and the patients’ written consent. Bone tissue from (potential) tumor patients or patients with viral or bacterial infections was excluded from the study.

Isolation and culture of primary human osteoblasts

We obtained femur heads from 8 patients (7 female, 1 male) with an average age of 75.25 years. Briefly, cancellous bone was removed mechanically from the femur head, washed 3–5 times with an average age of 75.25 years. Briefly, cancellous bone was removed mechanically from the femur head, washed 3–5 times with DPBS followed by 1 h incubation at 37°C with an equal volume of digestion buffer (DPBS, 0.07% Collagenase II – Biochrom AG, Berlin, Germany). After digestion, cancellous bone was washed with DPBS and transferred to cell culture flasks in culture medium (MEM/Ham’s F12, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml Streptomycin, 50 μM L-ascorbate-2-phosphate, 50 μM β-glycerol-phosphate). Medium was changed every 4–5 days. Within two weeks cells were growing out of the bone pieces [43,46]. Osteoblasts were cultured and expanded until passage 3, where a pure population of osteoblasts was reached, as determined by flow cytometry, negative for CD14 and CD45 and positive for CD90 and CD105. Only cells in passage 3 and 4 were used for the experiments. For differentiation, cells were cultured for up to 20 days with differentiation medium (MEM/Ham’s F12, 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml Streptomycin, 10 μM L-ascorbate-2-phosphate, 10 mM β-glycerol-phosphate, 25 mM HEPES, 1.5 mM CaCl2, 100 mM Dexamethasone). Medium was changed every 4th day.

Transient Cell Infections and Reporter Gene Assays

Cells were infected with the Sma/d1/4 reporter adenovirus (Ad5-BRE-Luc/provided from Prof. P. ten Dijke) or the Smad3/4 reporter adenovirus (Ad5-CAGA9-MLPLuc) as described before [47]. Upon binding of phosphorylated Smad1/4 or Smad3/4, respectively, luciferase if expressed in the cytoplasm of the cells. Cell lysates and luciferase activity measurement was done according to the manufacturer’s instructions, using the Steady-Glo Luciferase Assay System (Promega, Madison, USA) and normalized to total protein content.

Furthermore, we infected cells with adenoviral particles resulting in the expression of constitutive active Alk5 (Ad5-CA/Alk5), to investigate TGF-β effects independent of substrate binding. The expressed Alk5 is genetically modified in a way to constitutively activate Sma/d3/4 phosphorylation and associated signaling.

Infection efficiency was shown to be >90% by fluorescent microscopy of cells infected with Ad5-GFP (24 h).

Alkaline Phosphatase (AP) Activity Measurement

Prior to substrate incubation with pNPP buffer (0.2% 4-nitrophenyl-phosphate disodium salt hexahydrate, 50 mM glycine, 1 mM MgCl2, 100 mM TRIS, pH 10.5) for 1 h, cells were washed with DPBS. Resulting formation of 4-nitrophenol (pNP), was determined photometrically at 405 nm. Signal was normalized to relative cell number determined by Sulforhodamine (SRB) staining as reported [48].

Table 2. Summary of PCR conditions.

| Gene | GeneBank accession [NM.] | Forward Primer 5′-3′ | Reverse Primer 5′-3′ | Tm [°C] | Product length [bp] |
|------|--------------------------|----------------------|----------------------|--------|---------------------|
| AP   | 000478.3                 | ACG TGG CTA AGA ATG TCA TC | CTT GTA GGC GAT GTC CTT A | 53     | 476                 |
| ColI | 000088.3                 | CAG CCG CTT CAT CTA CAG C | TTT TGT ATT CAA TCA CTG TCT TGC C | 56     | 84                  |
| OC   | 199173.7                 | CCA GCC GTG TAG AGT CCA GC | GAC ACC CTA GA CCG GCC GT | 56     | 236                 |
| OP   | 000582                   | CCT CAT TGA CTC GAA CCA CTC | CGT CTG TAG CAT CAG GGT ACT G | 60     | 257                 |
| ON   | 003118                   | AGC ACC CCA TTG AGT GGT A | GGT CAC AGG TCT CGA AAA AGC | 60     | 105                 |
| BMP2 | 001200                   | CCC CCT ACA TGC TAG ACC TG | CAC TCG TTG CTG GTA GGT CTT CC | 60     | 150                 |
| BSP2 | 004967                   | TGA CTC ATC CGA AGA AAA TGG AG | CTG GTA GTC AGC TAA CCC TGT | 60     | 202                 |
| MGP  | 000900                   | AGA TGG AGA GCT AAA GTC CAA GA | GTA GGC TTC GCA AAG TCT GTA | 60     | 102                 |
| O5F2 | 006475                   | TAA GTT TCG TGG TAG TAC C | GTG TGG GTC TCT CAG TTT TGA TA | 60     | 140                 |
| OPG  | 002546.3                 | CCG GAA ACA GTG AAT CAA CTC | AGG TTA GCA TGT CCA ATG TG | 60     | 313                 |
| GAPDH| 002046.3                 | GTC AGT GGT GGA CCT GAC CT | AGG GGT CTA CAT GGC AAC TG | 54     | 420                 |

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Von Kossa and Alizarin Red Staining

Prior to fixation of cells with 100% ice cold ethanol (≥1 h), cells were washed with DPBS. Briefly, ethanol was removed by washing cells 3 times with tap water. For von Kossa staining cells were covered with staining solution (3% silver-nitrate) for 30 min at RT. To remove excessive staining solution, cells were washed 3 times with tap water. For color development (brownish-black) cells were covered with sodium-carbonate-formaldehyde solution (0.5 M sodium-carbonate, 10% formaldehyde). Pictures were taken with an inverted microscope. For Alizarin Red staining cells were covered with staining solution (0.5% alizarin red, pH = 4.0) for 30 min at RT. Excessive staining solution was removed by washing cells 3 times with tap water. Staining was resolved with sodium-carbonate, 10% formaldehyde). Washing cells 3 times with tap water. For von Kossa staining cells were removed by washing cells 3 times with tap water. Staining was resolved with 10% Cetylpyridiumchloride solution and resulting optical densities were measured at 562 nm [49]. Signals were normalized to relative cell number determined by Alamar Blue conversion (Biozol, Eching Germany).

Conventional RT-PCR

Total cellular RNA was isolated with Trifast (Peglah, Erlangen, Germany) according to the manufacturer’s protocol. First-strand cDNA was synthesized from 1 μg total RNA using the Transcriptor High Fidelity cDNA synthesis kit (Roche, Mannheim, Germany). Primer information are summarized in Table 2.

Statistics

Results are expressed as mean ± standard deviation of at least 3 independent experiments (N≥3) measured as triplicates or more (n≥3). Data sets were compared by one-way analysis of variance (Kruskal-Wallis) followed by Dunn’s multiple comparison test (GraphPad Prism Software, El Camino Real, USA). p<0.05 was taken as minimum level of significance.

Author Contributions

Conceived and designed the experiments: SE AKN. Performed the experiments: SE JB AS SD. Analyzed the data: SE JB AS MN ML SD BW AKN. Contributed reagents/materials/analysis tools: MN ML SD HV US AKN. Wrote the paper: SE JB AS MN ML HV BW US AKN.

References

1. Reddi AH (1998) Role of morphogenetic proteins in skeletal tissue engineering and regeneration. Nat Biotechnol 16: 247–252.
2. Bonefeld LF, Dallas SL (1994) Role of active and latent transforming growth factor factor beta in bone formation. J Cell Biochem 55: 350–357.
3. Erlebacher A, Filvaroff EH, Yr JQ, Derynck R (1998) Osteoblastic responses to TGF-beta during bone remodeling. Mol Cell Biol 9: 1903–1918.
4. Kopey PG, Young MF, Flanders KC, Roche NS, Koudaiah P, et al. (1997) Osteoblasts synthesize and respond to transforming growth factor-type beta (TGF-beta) in vitro. J Cell Biol 105: 457–463.
5. Pfeilschifter J, Bonevid L, Mundy GR (1990) Characterization of the latent transforming growth factor beta complex in bone. J Bone Miner Res 5: 49–58.
6. Noda M, Camilliere JJ (1989). In vivo stimulation of bone formation by transforming growth factor-beta. Endocrinology 124: 2991–2994.
7. Gressner AM, Weiskirchen R, Beinkopf K, Dooley S (2002) Roles of TGF-beta in hepatic fibrosis. Front Bioi 7: 679–807.
8. Kander S, Baumann M, Schumacher P, Dries V, Beyer E, et al. (2001) Prediction of progressive liver fibrosis in hepatitis C infection by serum and tissue levels of transforming growth factor-beta. J Viral Hepat 8: 430–437.
9. BORDER WA, Noble NA (1994) Transforming growth factor beta in bone formation. J Cell Biochem 55: 350–357.
10. Fedulov AV, Ses TP, Gvasirishva NA, Ryabaev MG, Vavyssva JG, et al. (2005) Serum TGF-beta 1 and TNF-alpha levels and chronic renal failure. Immunol Invest 34: 143–152.
11. Leak A (2007). TGF-beta, cardiac fibroblasts, and the fibrotic response. Cardiovasc Res 74: 207–212.
12. Derynck R, Zhang YE (2003) Smad-dependent and Smad-independent pathways in the TGF-beta family signalling. Nature 425: 577–586.
13. Shi Y, Massagué J (2003) Mechanisms of TGF-beta signaling from cell to nucleus. Cell 113: 685–700.
14. Pick E, Heldin CH, Ten Dijke P (1999) Specificity, diversity, and regulation in TGF-beta superfamily signaling. FASEB J 13: 2015–2124.
15. Miyazono K, Shinozaki M, Hara T, Furuya T, Miyazono K (2002) Two major transforming growth factor beta complex in bone. J Bone Miner Res 5: 49–58.
16. Inman GJ, Nicolas EJ, Callahan HF, Harling GD, Gaster LM, et al. (2002) Smad3 induces a potent and specific inhibitor of transforming growth factor-beta superfamily type 1 activator receptor-like kinase (ALK) receptors ALK1, ALK5, and AKT. Mol Pharmacol 62: 63–74.
17. Centrella M, Horowitz MC, Wozney JM, McCarthy TL (1994) Transforming growth factor-beta gene family members and bone. Endocr Rev 15: 27–39.
18. Schmidmayer G, Lucke M, Schwabe P, Raschke M, Haas NP, et al. (2006) Collective review: bioactive implants coated with poly(D,L-lactide) and growth factors TGF-I, TGF-beta1, or BMP-2 for stimulation of fracture healing. J Long Term Eff Med Implants 16: 61–69.
19. Wilmanns M, Schmidtmaier G, Breiner N, Huning M, Stange R, et al. (2004) Quantiﬁcation, localization, and expression of TGF-I and TGF-beta1 during growth factor-stimulated fracture healing. Calcif Tissue Int 74: 388–397.
20. Dallas SL, Sivakumar P, Jones CJ, Chen Q, Peters DM, et al. (2005) Fibronectin regulates latent transforming growth factor-beta (TGF-beta) by controlling matrix assembly of latent TGF beta-binding protein-1. J Biol Chem 280: 10871–10880.
21. Harris NE, Bonefeld LF, Harris MA, Sabatini M, Dallas S, et al. (1994) Effects of transforming growth factor beta on bone nodule formation and expression of bone morphogenetic protein 2, osteocalcin, osteopontin, alkaline phosphatase, and type I collagen mRNA in long-term cultures of fetal rat calvarial osteoblasts. J Bone Miner Res 9: 153–163.
22. Ignotz RA, Massague J (1987) Cell adhesion protein receptors as targets for transforming growth factor-beta action. Cell 51: 189–197.
23. Lind M (1998) Growth factor stimulation of bone healing. Effects on osteoblasts, osteocytes, and implants fixation. Acta Orthop Scand Suppl 283: 2–37.
24. Dieudonne SF, Fso P, van Zoden EL, Burger EH (1991) Inhibiting and stimulating effects of TGF-beta 1 on osteoclast bone resorption in fetal mouse bone organ cultures. J Bone Miner Res 6: 479–487.
25. Hattersley G, Chambers T (1991) Effects of transforming growth factor beta 1 on the regulation of osteoclastic development and function. J Bone Miner Res 6: 165–172.
26. Lamberts SW, van den Beld AW, van der Lely AJ (1997) The endocrinology of aging. Science 278: 419–424.
27. Manolas SC (2000) Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocrinology 21: 115–137.
28. Rosen CJ (1994) Growth hormone, insulin-like growth factors, and the senescent skeleton: Ponce de Leon’s Fountain revisited? J Cell Biochem 56: 348–356.
29. Wrana JL, Maemo H, Hawrylyshyn B, Yao KL, Domenicucci C, et al. (1988) Differential effects of transforming growth factor-beta on the synthesis of extracellular matrix proteins by normal fetal rat calvarial bone cell populations. J Cell Bioi 106: 915–924.
30. Geiger AG, Zeng QQ, Sato M, Heverling LM, Hirano T, et al. (1998) Decreased bone mass and bone elasticity in mice lacking the transforming growth factor-beta1 gene. Bone 23: 87–93.
31. Joyce ME, Jiangshi S, Belonder ME (1998). Transforming growth factor-beta in the regulation of fracture repair. Orthop Clin North Am 21: 199–209.
32. Rosen D, Miller SC, DeLeon E, Thompson AV, Bentz H, et al. (1994) Systemic administration of recombinant transforming growth factor factor beta 2 (TGF-beta2) stimulates parameters of cancellous bone formation in juvenile and adult rats. Bone 15: 355–359.
33. Erlebacher A, Derynck R (1996) Increased expression of TGF-beta 2 in osteoblasts results in an osteoporosis-like phenotype. J Cell Biol 132: 193–210.
34. Filvaroff E, Erlebacher A, Yr JQ, Gudeman SE, Lotz J, et al. (1999) Inhibition of TGF-beta receptor signaling in osteoblasts leads to decreased bone remodeling and increased trabecular bone mass. Development 126: 4267–4279.
35. Mohammad KS, Chen CG, Balooch G, Stebbins E, McKenna CR, et al. (2009) Pharmacologic inhibition of the TGF-beta type I receptor kinase has anabolic and anti-catabolic effects on bone. PLoS One 4: e5275.
36. Lai CF, Cheng SL (2002) Signal transductions induced by bone morphogenic protein-2 and transforming growth factor-beta in normal human osteoblastic cells. J Biol Chem 277: 15314–15322.
37. Rosier RN, O’Keefe RJ, Hicks DG (1998) The potential role of transforming growth factor beta in fracture healing. Clin Orthop Relat Res. pp S294–300.
38. Thirunavukkarasu K, Miles RR, Halladay DL, Yang X, Galvin RJ, et al. (2001) Stimulation of osteoprotegerin (OPG) gene expression by transforming growth factor-beta (TGF-beta). Mapping of the OPG promoter region that mediates TGF-beta effects. J Biol Chem 276: 36241–36250.
39. Kaneda T, Nojima T, Nakagawa M, Ogasawara A, Kaneko H, et al. (2000) Endogenous production of TGF-beta is essential for osteoclastogenesis induced by a combination of receptor activator of NF-kappa B ligand and macrophage-colony-stimulating factor. J Immunol 165: 4254–4263.
40. Maeda S, Hayashi M, Koniya S, Imamura T, Miyazono K (2004) Endogenous TGF-beta signaling suppresses maturation of osteoblastic mesenchymal cells. EMBO J 23: 532–533.
41. Boden SD, Kang J, Sandhu H, Heller JG (2002) Use of recombinant human bone morphogenetic protein-2 to achieve posterolateral lumbar spine fusion in humans: a prospective, randomized clinical pilot trial. 2002 Volvo Award in clinical studies. Spine (Phila Pa 1976) 27: 2662–2673.
42. Advincula MC, Rahemtulla FG, Advincula RC, Aida ET, Lemons JE, et al. (2006) Osteoblast adhesion and matrix mineralization on sol-gel-derived titanium oxide. Biomaterials 27: 2201–2212.
43. El-Amin SF, Botschwey E, Tuli R, Kofron MD, Mesfin A, et al. (2006) Human osteoblast cells: isolation, characterization, and growth on polymers for musculoskeletal tissue engineering. J Biomed Mater Res A 76: 439–449.
44. Harbers GM, Healy KE (2003) The effect of ligand type and density on osteoblast adhesion, proliferation, and matrix mineralization. J Biomed Mater Res A 75: 855–869.
45. Pfeilschifter J, Mundy GR (1987) Modulation of type beta transforming growth factor activity in bone cultures by osteotropic hormones. Proc Natl Acad Sci U S A 84: 2024–2028.
46. Robey PG, Termine JD (1985) Human bone cells in vitro. Calcif Tissue Int 37: 493–496.
47. Dooley S, Hamzavi J, Cioclan L, Godoy P, Ilkavets I, et al. (2008) Hepatocyte-specific Smad7 expression attenuates TGF-beta-mediated fibrogenesis and protects against liver damage. Gastroenterology 135: 642–659.
48. Skehan P, Storeng R, Scudiero D, Monka A, McMahon J, et al. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82: 1107–1112.
49. Wildemann B, Lubberstedt M, Haas NP, Raschke M, Schneidersmaier G (2004) IGF-I and TGF-beta 1 incorporated in a poly(D,L-lactide) implant coating maintain their activity over long-term storage-cell culture studies on primary human osteoblast-like cells. Biomaterials 25: 3639–3644.