

Cellular delivery of TGF β_1 promotes osteoinductive signalling for bone regeneration

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Abstract

Administration of osteoinductive growth factors to wound sites, alone or in conjunction with a delivery vehicle, is an appealing treatment option for critical bone defects. The delivery of cells transfected with genes encoding for osteoinductive growth factors, such as TGF β_1 , represents an attractive option to locally deliver constant levels of these growth factors to stimulate new bone formation at the defect site. Using non-viral transfection methods, we showed that osteoblasts can be genetically modified *in vitro* to secrete sustained therapeutic levels of TGF β_1 in its active form through control of the transfected cell environment. In addition, delivery of TGF β_1 produced by genetically modified cells that contained the proper post-translational modifications provided a more robust cellular response compared to administration of bacterially-derived recombinant TGF β_1 . Migration and subsequent proliferation of osteoblasts are critical aspects of the initial steps in the cascade of new bone tissue formation. Exposure to mammalian-derived TGF β_1 induced a more pronounced chemotactic response upon administration of 10 pg/ml TGF β_1 , whereas osteoblasts showed enhanced levels of metabolic activity at 100 pg/ml, which is indicative of greater levels of cellular proliferation when compared to addition of the same levels of recombinant TGF β_1 . This increased efficacy of cell-derived TGF β_1 over recombinant forms of TGF β_1 , combined with provision of a continual source of TGF β_1 , highlights the advantages of delivering genetically modified cells over exogenous protein delivery for bone tissue engineering. Copyright © 2007 John Wiley & Sons, Ltd.

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The regeneration and repair of damaged bone tissue involves the migration and proliferation of osteoprogenitor cells to the wound site, followed by the differentiation of these cells into mature osteoblasts. The mature osteoblasts then secrete and mineralize extracellular matrix (ECM), forming new bone. During the repair process, several osteoinductive signals, growth factors and cytokines stimulate the cells and regulate their activity (Bostrom and Asnis, 1998; Bostrom, 1998; Mundy *et al.*, 1995). While bone can regenerate new tissue without the formation of a scar, non-regenerative bone defects

can arise from trauma, disease, developmental deformities and tumours. Although the exact reason for the non-union of bone in these cases is unknown, several *in vivo* studies have shown that the exogenous delivery of osteoinductive signals can stimulate new bone formation in critical-sized defects (Einhorn, 1999; Govender *et al.*, 2002; Mackie and Trechsel, 1990). These results indicate that in at least some non-union fractures, the signals normally present during bone formation are absent or may only be present in concentrations too low to induce healing. Consequently, direct delivery of osteoinductive molecules to the wound site is an appealing treatment for non-regenerative bone defects to either enhance or replace current therapies, which typically involve bone grafts or fixation with internal or external implants. Transforming growth factor β_1 (TGF β_1) is perhaps one of the most important growth factors for bone formation and

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homeostasis. TGF β ₁ is the most prevalent growth factor found in the bone matrix, present at 100 times higher concentration in bone than in other tissues within the body, and the importance of this growth factor is evident from the large number of TGF β ₁ receptors present within osteoblasts (Bostrom and Asnis, 1998).

Several studies have shown that direct delivery of TGF β ₁ to the site of critical bone defects can stimulate new bone formation (Einhorn, 1999; Mackie and Trechsel, 1990); however, concerns surrounding protein instability and the extremely short half-lives of several growth factors necessitates multiple large-bolus delivery of proteins in order to achieve a sustained therapeutic concentration of protein (Chen and Mooney, 2003; Mahato *et al.*, 1999). Gene therapy represents an alternative to protein delivery that may be used to provide prolonged delivery of therapeutic concentrations of active forms of osteoinductive growth factors necessary for new tissue formation. By supplying the growth factor via gene therapy methods, one can circumvent the undesirable qualities of direct protein delivery, such as protein instability and the need for bolus delivery of multiple doses. Recent work has demonstrated the successful delivery of plasmid DNA from various different scaffold materials, such as collagen, PLGA, photopolymerizable PEG-based hydrogels and polyanhydrides (Bonadio, 2002; Bonadio *et al.*, 1999, 2002; Quick and Anseth, 2003, 2004; Quick *et al.*, 2004; Shea *et al.*, 1999). Other investigators demonstrated the successful healing of non-regenerative bone defects via the delivery of plasmid DNA embedded in collagen sponges (Bonadio *et al.*, 1999; Fang *et al.*, 1996; Patil *et al.*, 2000). Therefore, transfected cells have the potential to serve as local bioreactors and provide sustained production and delivery of TGF β ₁.

Transfection conditions for fetal rat osteoblasts (rOB) were optimized to provide conditions in which rOBs produced the highest level of TGF β ₁ as well as providing the highest degree of active TGF β ₁ secretion. During homeostasis, osteoblast-secreted TGF β ₁ is stored in the bone matrix in a latent form. Latent TGF β ₁ consists of the 25 kDa TGF β ₁ protein bound to a propeptide sequence known as the latent binding protein (LBP). The LBP inhibits binding of the protein to cell surface receptors, preventing TGF β ₁ from becoming a functioning osteoinductive signal. During normal tissue repair, osteoclasts secrete acidic products and metalloproteases that dissociate the LBP from TGF β ₁, thereby releasing the active form of the protein (Bostrom and Asnis, 1998; Mundy *et al.*, 1995). *In vitro*, acidification or heat treatment will dissociate the LBP from TGF β ₁, converting the protein to the active form (Lawrence *et al.*, 1984). Although the exact mechanism of activation *in vivo* is unknown, TGF β ₁ is released from the ECM in its active form at the site of bone resorption (Mundy *et al.*, 1995). Delivery of active TGF β ₁ is required for stimulation of new bone growth.

Third-passage osteoblasts isolated from fetal rat calvaria (rOBs) were transfected with FuGENE6TM (Roche)

cationic lipid-based non-viral formulations complexed with pCMV-SPORT-TGF β ₁ plasmid DNA at a transfection reagent ratio of 2 μ g DNA:3 μ l FUGENE reagent in DMEM supplemented with 0% or 5% FBS. Complexes containing 1 μ g of plasmid were then added to the rOBs. The culture medium was removed and replaced with fresh medium post-transfection at specified times, and the media was assayed for TGF β ₁ content using the TGF β ₁ E_{max} Immuno Assay (Promega), which detects only active TGF β ₁. The endogenous level of TGF β ₁ in the FBS stock used in all studies was 17 ng/ml and was accounted for in calculations for all sample analyses. Optimization of transfection conditions for fetal rat osteoblasts (rOB) with FuGENE vectors indicated that cells transfected in 5% FBS provided the highest level of TGF β ₁ production compared to non-transfected control cells at 72 h post-transfection, producing almost double the amount of TGF β ₁ (1322 \pm 252 pg/ml vs. 802 \pm 117 pg/ml, respectively). In addition, cells transfected and cultured in serum-free medium showed almost negligible levels of TGF β ₁ production compared to the control cell cultures 24 h post-transfection (150 \pm 4 pg/ml vs. 57 \pm 16 pg/ml TGF β ₁ expression, respectively). ELISA assays that recognize only the active form of TGF β ₁ showed that, on average, 9–13% TGF β ₁ produced by transfected rOB in 5% FBS was in the active form, whereas 74% TGF β ₁ produced by rOB in serum-free medium was in the active form. Although the higher fraction of TGF β ₁ produced in serum-free conditions is in the active form, the total amount of active TGF β ₁ is still significantly lower than the level present from transfection in 5% FBS (354 \pm 20.4 pg/ml vs. 690 \pm 68.5 pg/ml, respectively). Nevertheless, these data support the hypothesis that when exposed to stressful conditions, such as low-serum culture conditions, osteoblasts will spontaneously convert latent TGF β ₁ to the active form. This discovery may lead to the ability to control the degree of latent or active TGF β ₁ produced by manipulating culture conditions.

Migration and subsequent proliferation of osteoblasts are critical aspects of the initial steps in the cascade of new bone tissue formation. Several studies have shown that pg/ml concentrations of TGF β ₁ are able to induce various responses in osteoblasts, such as proliferation, migration and increased calcium production (Hughes *et al.*, 1992; Peter *et al.*, 2000; Pfeilschifter *et al.*, 1990; Reyes-Botella *et al.*, 2002; Zhang *et al.*, 2003). Although recombinant forms of TGF β ₁ can be produced in large quantities, proteins produced by mammalian cells become post-translationally modified and are theorized to induce a more robust response in comparison to recombinant TGF β ₁ (Bonadio, 2002). TGF β ₁ is a potent osteoinductive signal capable of stimulating both proliferation and migration of bone-forming cells. Concentrations of the growth factor in the range from 10–1000 pg/ml have been shown *in vitro* to have chemotactic effects on osteoblasts, depending on the source of TGF β ₁ (Hughes *et al.*, 1992; Pfeilschifter *et al.*, 1990). Hughes *et al.* (1992) showed that a maximal chemotactic response of rOB occurred when the cells were exposed to 100

pg/ml recombinant TGF β_1 . In contrast, Pfeilschifter *et al.* (1990) found that addition of active mammalian forms of TGF β_1 to rOBs at concentrations of 5–15 pg/ml were optimal for cell migration. Dramatic differences in the promotion of migration between cellular (rOB) and recombinant sources (bacterial) of TGF β_1 were also seen at lower protein concentrations, with the highest level of chemotaxis observed at 10 pg/ml cell-derived TGF β_1 . These results indicate that cells will respond in a more robust fashion to stimulants produced by an allogenic source of TGF β_1 compared to a recombinant source.

A modified Boyden chamber assay was used to determine cell migration in response to TGF β_1 . Briefly, serum-free DMEM spiked with 0–100 pg/ml of either recombinant (Peprotech) or mammalian cell-secreted TGF β_1 was added to the bottom of a 24-well transwell plate (8 μ m diameter pores within inserts; Corning). rOB suspensions containing 1×10^5 and 2×10^5 cells/ml were added to the top of each insert, and the number of cells that migrated through the bottom of the insert, towards the higher concentration of TGF β_1 , as well as those that remained within the insert, were counted using a Coulter counter. The mammalian TGF β_1 was obtained from NIH3T3 fibroblasts transfected and cultured in 5% FBS in a fashion similar to rOBs, as described previously. When rOBs were exposed to varying concentrations of either mammalian or recombinant TGF β_1 , the chemotactic response to the mammalian cell products was significantly greater than the response to the recombinant protein at all levels of TGF β_1 tested (Figure 1). Thus, by delivering plasmid and/or *ex vivo* transfected cells, the necessary therapeutic doses may be lower, due to the more robust response that cell-secreted proteins are capable of achieving.

In addition to being a chemotactic agent, TGF β_1 has been shown to induce cell proliferation (Mundy *et al.*, 1995; Peter *et al.*, 2000; Reyes-Botella *et al.*, 2002). Cellular proliferation was assessed indirectly by monitoring general cellular metabolic activity in response to exposure to TGF β_1 produced by both recombinant and mammalian

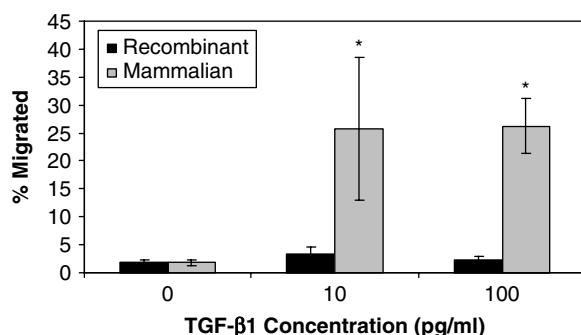


Figure 1. Chemotactic response of rOBs to either recombinant (bacterial) or mammalian (transfected NIH3T3 cells) sources of TGF β_1 . The percentage of cells migrated represents the fraction of cells translocated through trans-well inserts towards the higher concentration of TGF β_1 added to the bottom of the trans-well plate. Error bars represent 1 SD from three replicates. * $p \leq 0.1$, as determined by Student's *t*-test

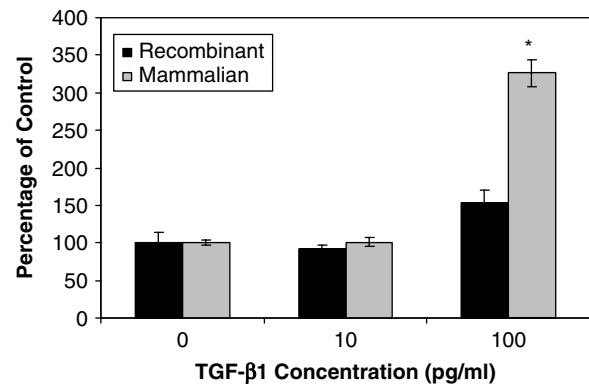


Figure 2. Comparison of rOB metabolic activity in response to medium supplemented with TGF β_1 from either recombinant or mammalian sources. The studies were conducted in serum-free medium. The data were normalized to the metabolic activity from rOBs that were not supplemented with TGF β_1 , which was set at 100%. Error bars represent 1 SD from three replicates. * $p \leq 0.03$, as determined by Student's *t*-test

sources. 100 μ l of either recombinant or mammalian cell-secreted TGF β_1 -spiked serum-free DMEM was added to rOBs at 0 or 100 pg/ml TGF β_1 for 3 days before determining metabolic activity, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay, and the absorbance of each sample was measured at 560 nm. Figure 2 shows that exposure of rOBs to mammalian-secreted TGF β_1 significantly enhanced the metabolic activity of rOBs compared to recombinant TGF β_1 . These results indicate that mammalian-produced TGF β_1 can enhance metabolic activity within rOBs, which can translate to increased proliferation of these cells.

Delivery of TGF β_1 via gene therapy methods, therefore, represents a promising therapeutic for the treatment of non-regenerative bone defects. We have demonstrated the ability to transfect rOBs to produce the active form of TGF β_1 , and that a more robust response is attained with two critical aspects of new bone formation, cellular migration and proliferation, using mammalian-generated TGF β_1 compared to bacterially produced TGF β_1 . By delivering genetically modified cells, continual translation of the delivered genetic sequence will result in a sustained production of active forms of TGF β_1 , which may help promote bone regeneration in otherwise non-regenerative fractures.

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