The effect of transforming growth factor β1 (TGF-β1) on the regenerate bone in distraction osteogenesis

A biomechanical, histologic and immunohistochemical study on rabbits

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Abstract

Distraction osteogenesis is a well established clinical treatment for limb length discrepancy and skeletal deformities. Transforming growth factor beta 1 (TGF-β1) is a multifunctional peptide which controls proliferation and expression of cells specific to bone like chondrocytes, osteoblasts, osteoclasts including mesenchymal precursor cells.

To decrease the external fixation time with increasing the strength of regenerate (newly formed bone after distraction) we tested the effect of locally applied transforming growth factor beta 1 on distraction osteogenesis.

A total of 28 mature female white New zealand rabbits weighing 3.5 kg–4.5 kg were studied. 10 animals were belonging to biomechanical testing group (5 for the study and 5 for the control subgroups), and the others were to histology group. In biomechanical group after tibial osteotomy TGF-β1 was applied subperiosteally for 5 days just proximal to osteotomy site. Control group received only the solvent. Seven days after tibial osteotomy distraction was started at a rate of 0.25mm/12 hours for 3 weeks with a unilateral fixator. Rabbits were sacrified at the end of a consolidation period 8 week after tibial osteotomy.

We assessed density of the elongation zone of rabbit tibial bones with the computed tomography. Then biomechanical parametres were assessed using the torsional testing using the material testing machine. In histology group rabbits were classified as control and study (rabbits that were given TGF-β1). Rabbits were sacrified at the end of first week, second week and fourth week also at the end of consolidation period 8 week after tibial osteotomy. Immunohistochemical and histologic parameters were examined.

Biomechanical testing was applied as torsional testing. These values are used in determination of maximal loading, stiffness and energy absorbed during testing (brittleness). The histomorphometric examination looked for the differences between the study and control groups in terms of bone formation pattern, bone quality and quantity. The immunohistochemical studies investigated the mechanism of TGF- β1, and it’s presence in different cell types.

The results of this study suggest that locally applied TGF-β1 improves the mineral density of distraction gap and load to failure (energy absorbed during testing). Though there is no significant histomorphometric difference between the study and control groups, there is an increased bone mineral density and an according maximum energy absorbance in the study group.

This effect can be explained by the following mechanism:

TGF-β1 exerts it’s effect on two different receptor types (Type 1 and 2). Type 1 receptors are localized to bone matrix and type 2 receptors are localized to the intracellullar space. The specific stains utilized in the current experiment are specific to type 2 receptors. They have been shown to be down-regulated by exogenous TGF-β1 injections. Most probably, type 1
receptors are up-regulated by this exogenous administration, but unfortunately, there is currently no specific stain on the market to display type 1 receptors and to prove this explanation.

**Introduction**

Limb lengthening procedures are usually performed with external fixators, relying on the rules of distraction osteogenesis. Surgical procedures making use of distraction osteogenesis are also applied to the treatment of segmentary bone defects. Lengthening and bone transport procedures, though with high success rates, are time consuming. In addition, the newly formed bone might be of poor quality, depending on the underlying pathology (Aronson 1993; Fischgrund et al. 1994).

A long treatment time and potential for poor regenerate are disadvantages of external fixation. Currently, many studies are conducted to find biologic tools which should decrease the external fixation period and to increase the quality of the regenerate bone.

TGF-β1 is the most abundant growth factor of the bone matrix. This multifunctional polypeptide has a broad range of cellular activities including control of proliferation and expression of the differentiated phenotype of several cell types specific to bone, among them mesenchymal precursor cells, chondrocytes, osteoblasts and osteoclasts (Joyce et al. 1990; Paley 1990).

TGF-β1 also plays a critical role in bone remodeling, and stimulates bone matrix protein synthesis (Centrella et al. 1991; Takeuchi et al. 1993). This growth factor has a proven effect in increasing callus volume and bending stiffness in experimental fracture models (Centrella et al. 1991).

Saadeh et al. reported on the positive effect of TGF-β1 on expression of osteoblastic vascular endothelial growth factor, thus upregulating fracture healing and bone formation (Bonewald and Mundy 1990). TGF-β1 is also showed to heal critical size skull defects in animals (Nielsen et al. 1994).

There is only one study in the literature investigating the effect of TGF-β1 on distraction osteogenesis (Saadeh et al. 1999). This experimental study has not been able to show any beneficial effect of TGF-β1.

The aim of our experimental study is to investigate the effect of TGF-β1 on the amount and quality of regenerate bone formed during bone distraction. Besides, our immunohistochemical examinations intend to display the pathway of TGF-β1 on specific receptors. In contrary to the single report in the literature, we utilized larger amounts of TGF-β1, and performed the injections during the latent period instead of the distraction period. In order to prepare a more mature callus to lengthen. This change of injection of TGF-β1 during latent period instead of distraction period depends on the ability of TGF-β1 to transform mesenchymal cells into osteoblasts (Paley 1990) and stimulate the proliferation of cells of osteogenic lineage at the distraction gap in order to prepare a more mature callus to lengthen. TGF-β1 is known to be an orthotopic agent, that is why we injected the subperiosteal space proximal to the osteotomy site, instead of the elongation gap.

**Material and methods**

A total of 28, skeletally mature, 20 months old, female, white, New Zealand rabbits weighing 3.0–3.5 kg were studied. The animals were randomly allocated into two groups. Ten rabbits were included into the biomechanical testing group and the rest into the histologic and immunohistochemical study group. Both groups were subdivided into TGF-β1 injected and non-injected groups. The animals were kept in 14 separate cages measuring 3 × 1 × 0.5 m, which allows them to freely ambulate. The surgical procedures and distraction protocols were approved by the university animal research ethics committee.

**External fixators**

A specially designed, monolateral external fixator, 10 cm long and 1 cm wide, with two clamps, each including slots for two specially designed Schanz screws, was utilized (Figures 1, 2). The distractor had a lengthener, which distracted one millimeter in a full turn. Kirschner wires of 2 mm diameter, with sharp,
self drilling, self tapping threads on the tip, were prepared.

**Surgical procedure**

The rabbits were anesthetized by intramuscular administration of ketamin (40 mg/kg, Ketalar®) and continued by 2 m³/min inhalation of sevorane (Sevorane®).

The right lower extremity of the animals were shaved and steriley prepared for the surgical procedure. Four specially designed Schanz screws were inserted, two above and two below the osteotomy site, each perpendicular to the sagittal and parallel to the frontal plane. The screws were inserted manually with T-handles following a predrilling with one millimeter K-wires (Figure 3).

The middiaphysis was exposed by sharp dissection. The periosteum was opened longitudinally. The osteotomy site was prepared with multiple, 1 mm, drill holes and then a low energy, transverse osteotomy was performed (Beck et al. 1993; Rauch et al. 2000). The osteotomy site was chosen to be just below the tibiofibular junction (Figure 4).

The periosstem and surrounding soft tissues were sutured by 4/0 cat-gut and the skin was closed by 3/0 cat-gut. The Schanz screws and the sutured area were covered by sterile, compressive dressing. The animals were allowed to freely ambulate in their cages immediately. Antibiotics (cefazoline-Na, 20 mg/kg, Sefazol®) were injected intramuscularly preoperatively and once a day for three days thereafter.

**Biomechanical study group.** The biomechanical study group consisted of 10 rabbits, 5 for the study and 5 for the control subgroups. Following a latent period of 1 week, distraction was initiated with 0.5 mm per day, divided into two equal increments, and performed for 3 weeks. At the end of the third week, distraction was stopped, the clamps were locked, and the consolidation period of 4 weeks started. During the latency period, the study animals were injected with 100 ng TGF-β1 (Sigma-Aldrich), subperiosteally, immediately proximal to the osteotomy, by a 27 gauge needle, once a day for 5 days.

At the end of the consolidation period, all rabbits were sacrificed by a high dose intravenous thiopentone-Na (pentothal®) injection following induction anesthesia with 40 mg/kg intramuscular ketamine injection. Computerized tomography scans of the distraction gap were taken. The bone density was measured by quantitative tomography scans. The cross sectional area in the middle of the distraction gap, was selected (ROI: region of interest), the bone density measured and recorded in Hounsfield units (Figure 5).

The external fixators were removed. After soft tissue dissection, the tibias were disarticulated from the knee and ankle joints, and the samples put into the Ringer’s solution (4°C, pH 7.4).

The samples were transferred to the Siemens A.G. mechanical study laboratory within 3 h. The sample ends, both proximally and distally, were embedded in polymethylmethacrylate (PMMA). For this purpose, specially designed plexiglas molds were used.
Approximately 10 mm at each end of the rabbit’s tibia was inserted at the center of the plexiglas mold. Then, the liquid PMMA was poured into the mold. The prepared samples were mechanically tested by The Material Testing Machine (Siemens A.G., Germany). This machine is able to examine the specimens for torsional, axial and bending stresses. We examined our specimens by the torsion test and measured the stiffness, strength and maximum energy absorbance capacity of the regenerate bone. The reason to choose the torsion test relies on the fact that during our everyday activities, our lower limb faces mostly torsion stresses.

**Histologic and immunohistochemistry group.** Eighteen rabbits were included in the histologic study group. During the latency period, study group were injected with 100 ng TGF-β1 (Sigma-Aldrich), subperiosteally, immediately proximal to the osteotomy, by a 27 gauge needle, once a day for 5 days. Control group received only the solvent. Control and study animals were sacrificed, at the end of the latent period (week 1), at the end of the first distraction week (week 2) and at the end of the consolidation period (week 8). Slides were prepared for histomorphometric and immunohistochemical studies. The histomorphometric examination looked for the differences between the study and control groups in terms of bone formation pattern, bone quality and quantity. The immunohistochemical studies try to investigate the effect of TGF-β1 on its receptors and it is presence in different cell types.

For immunohistochemical tests, TGF-RII (L-21):sc-400-G (TGF-β1 receptor antagonist), TGF-β1 (V):sc-146-G (TGF-β1 antagonist) (Santa Cruz Biotechnology, INC), and goat immunocruz staining system:sc-2053 (Santa Cruz Biotechnology, INC) were used. TGF-β1 (V) stain, which is an antibody to TGF-β1, was utilized to show the accumulation of TGF-β1 in various cell types.

For histomorphometric examination, 4–5 μm slices were prepared from paraffin blocks and stained with hematoxyline and eosin.

Localization of TGF-β1 and TGF-RII was performed using the indirect immunoperoxidase system. Stains and anti-stains were applied and the slides were examined by light microscopy (Sporn et al. 1986; 1987; Sferra et al. 1995; Eralp et al. 2004).

**Statistical analysis.** The values for bone mineral density were compared by unpaired T-test and Mann–Whitney U test. Values of maximum density, torsional stiffness, strength and maximum energy absorbance were compared by the Mann–Whitney U test. P values lower then 0.05 were accepted as statistically significant for a 95% confidence interval.

**Results**

**Histomorphometric and immunohistochemical results**

Light microscopy findings indicate that the main bone formation mechanism during distraction in both groups is intramembranous. Beside periosteal and endosteal osteoblasts, reactive chondrocytes also appeared with the initiation of distraction.

The findings according to phases of the experiment in both groups can be summarized in the following:

**Latency period.** The osteotomy front is covered with osteoblasts. The osteotomy site is filled with hematoma and a fibrous exudate.

**Distraction period (second week).** The hematoma and fibrous exudates is invaded by a large amount of capillaries.

**Distraction period (third and fourth week).** Bone trabecules formed at the osteotomy front reach the central zone of the distraction gap.

**End of consolidation period (eighth week).** Mature bone trabecules surrounded by irregular chondrocytes are depicted.

Hematoxylene and eosin stains show an especially active medullary and periosteal osteogenesis during the first 2 weeks. Periosteal callus tissue was more pronounced then endosteal bone formation. Though the newly formed bone was prominently of intramembranous type, the elongation gap contained some chondrocytes and fibrocartilaginous tissue, too.
This light microscopic examination displayed no histomorphometric difference between the two study groups.

The results of immunohistochemical staining for TGF-β1 and its receptors, according to experiment groups and study timing are summarized in Tables I and II.

Immunohistochemical stains revealed similar results in both experiment groups, depicting same kind of cells in both groups during all periods of the distraction osteogenesis with alike TGF-β1 expression in the cells of osteogenic lineage and bone matrix. TGF-β receptor type 2 stains showed a significant difference, displaying a downregulation of the TGF-β1 type 2 receptors in the TGF-β1 injected group comparing to control group (Figures 6, 7).

<table>
<thead>
<tr>
<th>Table I. Immunhistolocalisation of TGF-β1.</th>
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<td>Bone matrix</td>
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<td>C</td>
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<td>Latent period</td>
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<td>Distraction period</td>
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<td>Consolidation period</td>
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C, control group; S, study group; ++++, powerful; ++, fair; +, poor; +/−, very poor.

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<th>Table II. Immunhistolocalisation of TGF-β1 receptors.</th>
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<td>Bone matrix</td>
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<tr>
<td>C</td>
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<td>Consolidation period</td>
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C, control group; S, study group; ++++, powerful; ++, fair; +, poor; +/−, very poor.

Mechanical study results

All animals had serial radiologic control of their operated limbs every week, and after sacrifice. The distraction gaps were examined for bone mineral density using computerized tomography at the end of the consolidation period. The mean value of maximum bone density in the control group was 862.2 Hounsfield Units (minimum 410, maximum 1250) and in the study group 1437.8 Hounsfield Units (minimum 1140, maximum 1647). Unpaired T-test showed a statistically significant difference between the study and control groups (p: 0.04).

The distracted bone segments embedded in PMMA on both sides, were tested in the Material Testing Machine for rotational stiffness. The machine was adjusted to initiate torsion at 5°/s, gradually increasing to 50°/s within 10 s. The machine digitally recorded the toughness of the regenerate in Newton/mm² until failure occurred. The data were plotted into torque-rotation curves using AutoCad V4.0 software. Maximum torque and stiffness were calculated using these curves and maximum energy absorbance was calculated as the area under this curve.

The results are summarized in Table III. The only statistically significant difference was detected in maximum energy absorbance between the two groups, which correlated with the significantly increased maximum bone density in the study group.

Discussion

Methods using principles of distraction osteogenesis are widely used for the treatment of leg length discrepancies, bone defects and non-unions. Beside it
is revolutionary contribution to orthopedic surgery, treatment with external fixation have a disadvantage of long external fixation periods, well above one month for each centimeter of newly formed bone to heal.

Various approaches have been tested to accelerate osteogenesis such as: electrical stimulation (Steinberch et al. 2000), mechanical compression (Pepper et al. 1996), systemic treatment with growth hormone and vitamin-D analogs (Yamane et al. 1995) and local treatment with fibroblast growth factor-2 (Okazaki et al. 1999). Some of these studies have yielded promising results, but as of yet none of these approaches have been shown to be efficient in humans.

Mechanical test results yield an increased energy absorbance capacity of the new bone in the study group, which indicates a decreased brittleness but no difference regarding torsion and stiffness values between the two groups. Although computerized tomography scans showed a significant increase of the bone mineral density in the elongation gap, the results of the current experimental study show minimal effect of TGF-β1 on new bone formation.

Various studies in the literature indicate the beneficial effect of TGF-β1 in experimental bone defect and fracture healing models (Bonevald and Mundy 1990; Nielsen et al. 1994).

Our starting point for this experimental study was the proven positive effect of TGF-β1 on fracture healing and bone formation. In the literature, we could find only one study dealing with the impact of locally applied TGF-β1 on distraction osteogenesis (Saadeh et al. 1999). This study could not detect any positive effect. Some histological studies showing the increased endogenous TGF-β1 expression during distraction osteogenesis encouraged us. Eingarnertner et al. detected higher amount of TGF-β in the center and less on the periphery of the regenerate bone (Lind et al. 1993). Yeung et al. (2002) conducted an immunohistochemical study and concluded that TGF-β1 may play a role in transducing mechanical stimulation to biological tissue during distraction osteogenesis (Eingarther et al. 1999); Solheim 1998.

TGF-β1 is a multifunctional protein, regulating cell proliferation, differentiation and various cell functions (Paley 1990). It stimulates intramembranous and endochondral bone formation by increased formation of mesenchymal stem cells and their change into chondroblastic and osteoblastic cells (Joyce et al. 1990; Paley 1990).

Though bone morphogenetic proteins can show their effect heterotopically, TGF-β1 has only an orthotopic effect (Lind et al. 1993). Joyce et al. displayed a transformation of periosteal mesenchymal cells into chondroblasts and osteoblasts following subperiosteal injection of TGF-β1 in rats (Paley 1990).

Rauch et al. injected the elongation gap in their experimental study but they found no detectable effect on bone mineral density or histologically determined bone volume in the distraction gap. But it increased the amount of fibrous tissue in the regenerate region (Saadeh et al. 1999). This orthotopic effect led us to inject the subperiosteal area just proximal to the osteotomy, which should produce the osteoblasts necessary for the regenerate.

The histomorphometric and immunohistochemical components of the current study could not display any significant difference of bone formation pattern and amount between the study and control groups. In the immunohistochemical examination, there was no detectable staining change of endogenous TGF-β1 in control animals, and staining of exogenous and endogenous TGF-β1 and it is distribution in the cells localized to the distraction gap in study animals. Nevertheless, the TGF-β1 receptors were down-regulated in the study animals following exogenous TGF-β1 injection. This downregulation of the specific

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<th></th>
<th>Control</th>
<th>Study</th>
<th>Mean (minimum–maximum)</th>
<th>$P$-value (Mann–Whitney $U$)</th>
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<tbody>
<tr>
<td>Stiffness (N/mm$^2$)</td>
<td>Control</td>
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<td></td>
<td>Study</td>
<td>17.10 (13.62–21.11)</td>
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<td>Maximum energy absorbance</td>
<td>Control</td>
<td>570 (452–665)</td>
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<tr>
<td></td>
<td>Study</td>
<td>1531 (981–1914)</td>
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<tr>
<td>Strength (N)</td>
<td>Control</td>
<td>1512.6 (1293–1782)</td>
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<td></td>
<td>Study</td>
<td>1901.3 (1373–2329)</td>
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receptors explain the ineffectiveness of exogenous TGF-β1 on the cells in the distraction gap.

Though there is no significant histomorphometric difference between the study and control groups, there is an increased bone mineral density and an according maximum energy absorbance in the study group. This effect can be explained by the following mechanism:

TGF-β1 exerts its effect on two different receptor types (Type 1 and 2). Type 1 receptors are localized to bone matrix and Type 2 receptors are localized to the intracellular space. The specific stains utilized in the current experiment are specific to Type 2 receptors. They have been shown to be down-regulated by exogenous TGF-β1 injections. Most probably, Type 1 receptors are up-regulated by this exogenous administration, but unfortunately, there is currently no specific stain on the market to display Type 1 receptors and to prove this explanation.

Conclusion

The current study shows a positive effect of exogenous TGF-β1 application on the mineralization of the newly formed bone during distraction osteogenesis. Although this increased maximum energy absorbance capacity which is most probably related to the upregulation of Type 1 TGF-β1 receptors in the bone matrix, torsion and stiffness values showed no differences between the two groups. Overall, there was a minimal effect of TGF-β1 on new bone formation.

References


Transforming growth factor