Local injection of TGF-ß increases the strength of tibial fractures in the rat

Hanne M Nielsen[1], Troels T Andreassen[1], Thomas Ledet[2] and Hans Oxlund[1]

The effect of Transforming Growth Factor ß (TGF-ß) administered locally around the fracture line of healing rat tibial fractures was investigated after 40 days of healing. TGF-ß in a dose of 4 ng or 40 ng was injected every second day during the healing period. The strength, stiffness, energy absorption and deflection of the fractures were measured in a materials-testing machine. Compared with placebo-treated animals, the ultimate load of the fractures increased in the group injected with 40 ng of TGF-ß, but not in those injected with 4 ng. TGF-ß induced a dose-dependent increase in the cross-sectional area of the callus and bone at the fracture line. Consequently, local treatment of fractures with TGF-ß increases the callus formation and strength. The energy absorption and deflection capacities of the healing fractures are preserved.

University of Aarhus [1]*Department of Connective Tissue Biology, Institute of Anatomy; and* [2]*Research Laboratory for Biochemical Pathology, Institute of Experimental Clinical Research, The Municipal Hospital of Aarhus, Denmark*
Correspondance: Dr. Troels T Andreassen, Department of Connective Tissue Biology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus C, Denmark. Tel +45–89 423018. Fax –86 137539
Submitted 92-12-26. Accepted 93-09-19

Fracture repair is a complex process involving both systemically—and locally—produced factors (Bak and Andreassen 1991, Joyce et al. 1991). The locally produced Transforming Growth Factor ß (TGF-ß) has attracted considerable attention as being a local regulator of bone formation and resorption, both in relation to intact bones and healing fractures. TGF-ß1 and TGF-ß2 injections around the femoral bone of rats resulted in increased formation of both cartilage and bone (Joyce et al. 1990a). The localization and expression of TGF-ß have been investigated in rat femur fractures (Joyce et al. 1990b, c). By using immunohistochemistry, they showed both extra- and intracellular localization of TGF-ß during the different phases of healing. They also found that TGF-ß mRNA was apparent during the healing. In a dose-response study, Beck et al. (1991) has shown that TGF-ß1 induces rapid bone closure of skull defects. We report how local application of TGF-ß around a healing tibial fracture increases the mechanical strength of the fracture after 40 days of healing.

Animals and methods

Female Wistar rats (Møllegård, Lille Skensved, Denmark) 3-months-old at the time of fracturing were used. The rats were housed in cages in groups of 3, with 12/12 hour light-dark cycles and had free access to tap water and pellet food (Altromin Diet 1314, Christian Pedersen, Ringsted, Denmark).

Using pentobarbital (50 mg/kg i.p., SA, Copenhagen, Denmark) anesthesia, the operation was performed under sterile conditions. A standardized, closed fracture was produced above the tibiofibular junction in the right tibiae by 3-point bending (Bak and Andreassen 1988, 1989). Closed medullary nailing was performed with a 0.79 mm Kirschner wire, and the skin was closed with monofilament sutures. Contact radiographs were taken immediately after operation, and animals with fractures located less than 2 mm or more than 6 mm above the tibiofibular junction or with displaced wires were excluded. Unrestricted weight bearing was allowed; the rats resumed normal activity immediately after recovery from the anesthesia.

Local injection of TGF-ß around the fracture

After radiography, the animals were divided randomly into 3 groups and injected every second day around the fracture with: 1) placebo, 2) TGF-ß 4 ng, 3) TGF-ß 40 ng.

TGF-ß was obtained from human blood platelets (Assoian 1987). The isolated TGF-ß was analyzed on HPLC using reverse-phase chromatography in an acetonitrile gradient (C18VYDAC 218TP54 column, Vydac, Heseria, CA, U.S.A.). The position of the isolated TGF-ß was compared with a commercial prepar-

ation of TGF-ß (T-1654, Sigma, St. Louis, MO, U.S.A.). The protein concentration was calculated by the differences in the absorbance at 215 and 225 nm. The biological effect of the TGF-ß was tested in a tissue culture system of human arterial smooth muscle where the cell proliferation and collagen production were estimated.

The placebo group was injected with vehicle, 0.15 M saline with 0.2% rat albumin (Sigma, St. Louis, MO, U.S.A.) and 0.001 N HCl, pH 5.0. The TGF-ß preparations were solubilized in the vehicle, 1.33 µg/mL for the group injected with 40 ng/injection and 0.13 µg/mL for the group injected with 4 ng/injection. The vehicle and TGF-ß plus vehicle preparations were stored at –80 °C in vials at the start of the experiment and blinded with respect to the person who performed the injections.

Starting on the day of operation, the animals were injected every second day throughout the entire healing period of 40 days. To ensure that TGF-ß was injected exactly around the fracture site, the dermis around the fracture line was marked with Indian ink on the day of operation. The fracture line was identified by palpation under guidance of the contact radiographs. Before injection, the rats were anesthetized by intraperitoneal injection of a combination of methohexital (50 mg/kg, Eli Lilly & Co, Indianapolis, IN, U.S.A.) and tert-Amyl alcohol (0.14 mL/kg, Merck, Darmstadt, Germany), and the skin over the fracture was disinfected by 0.5% chlorhexidine in 62% ethanol (SA, Copenhagen, Denmark). 15 µL were injected in both the anteromedial and the anterolateral surfaces of the fracture.

Mechanical tests

Both the right fractured and the left non-fractured tibiae were tested after 40 days of healing. The rats were killed by an overdose of pentobarbital (250 mg/kg i.p.), and both tibiofibular bones were dissected free and stored in Ringer's solution (4 °C, pH 7.4) until testing, which was performed within 4 hours. Contact radiographs of all the fractures were obtained. The fibula and the proximal epiphysis were resected and the intramedullary nail was removed. The mechanical properties of the healing fractures were analyzed using a destructive 3-point-bending procedure. The bone was placed on 2 rounded bars at a distance of 15 mm in a materials-testing machine (Alwetron 250, Lorentzen and Wettre, Stockholm), and deflected from above by another rounded bar at the fracture line. A constant deflection speed of 2 mm/min was used. All the bones were oriented alike, with the concave facet of the lateral tibial condyle resting on one of the supporting bars and the bone loaded from the medial side. The left unfractured tibia was tested at the same level of the bone as that of the fracture in the corresponding right tibia, using exactly the same procedure. The load and deflection were recorded continuously by transducers coupled with measuring bridges, and the signals were fed to an x–y recorder. The load-deflection curves obtained were read by a digitizer into a calculator, and the following parameters were calculated: ultimate load, ultimate stiffness, deflection at ultimate load, and energy absorption at ultimate load (Andreassen et al. 1981, Bak et al. 1991). Before testing, the external transverse and anteroposterior diameters of the fracture were measured at the point of loading, using a sliding caliper. Likewise, dimensions of the non-fractured tibia were measured at the corresponding level. The transverse diameter of the marrow space was measured from the contact radiographs in a projection microscope, using the diameter of the nail as a reference. Stress values could then be calculated from the bending moment and the second moment of area (Kenedi 1980). In the non-fractured tibia, Young's modulus was calculated from ultimate stiffness, from the distance between the supporting bars in the bending procedure, and from the area moment of inertia, assuming that (a) the cross-sectional area of the bone was constant during loading, (b) the shape and area of the cross-section were constant between the supporting bars, (c) the extent of deflection was small, and (d) the composition of the bone was homogeneous (Kenedi 1980).

After mechanical testing, the cross-sectional area of the bone, including callus, was measured at the fracture line. A transverse section was cut by means of a bone saw (EXAKT-Apparatebau, Otto Hermann, Norderstedt, Germany). The sections were projected onto a screen by means of a projection microscope (Allen Microfilm Products, Bournemouth, UK) with ×21 magnification. The projected sections were outlined, and the inner and outer diameters were measured. Finally, the outlined sections were cut out and the cross-sectional areas estimated by their weight.

Of the 52 rats included in the experiment 17 were excluded; 7 died due to the anesthesia given in relation to local injections, and 10 had infection around the nail. The TGF-ß injections did not influence the body weight during the experiment.

Statistics

The data from the groups were analyzed by the non-parametric Kruskal-Wallis test and, in case of differences, the individual groups were compared with each other by the non-paired Mann-Whitney test. $P < 0.05$ was statistically significant.

Table 1. The effect of local injection with TGF-ß around the fracture line on the mechanical properties of healing tibial fracture in the rat. Mean, *SEM*

Experimental groups	n	Ultimate load (N)	Ultimate stress (N/mm^2)	Ultimate stiffness (N/mm)	Energy absorption at ultimate load (N × mm)	Deflection at ultimate load (mm)
Placebo	13	17 *3*	9.0 *1.7*	66 *16*	5.8 *1.1*	0.68 *0.11*
TGF-ß 4 ng/injection	12	18 *3*	6.8 *1.1*	55 *15*	6.0 *1.1*	0.65 *0.10*
40 ng/injection	10	31 *5*[a,b]	14.9 *6.1*	108 *44*	11.0 *3.1*	0.68 *0.11*
P-value (Kruskal-Wallis test)		0.02	0.3	0.6	0.2	0.9

Mann-Whitney's non-paired test. [a]$P < 0.01$ versus placebo; [b]$P < 0.05$ versus TGF-ß (4 ng/injection).

Table 2. Cross-sectional area of callus and bone at the fracture line. Mean *SEM*

Experimental groups	n	Callus-bone area (mm^2)	Medullary area (mm^2)
Placebo	13	12.9 *1.0*	4.1 *0.4*
TGF-ß 4 ng/injection	12	16.8 *1.3*[a]	5.0 *0.6*
40 ng/injection	9	19.5 *2.8*[b]	5.0 *0.9*
P-value (Kruskal-Wallis test)		0.04	0.4

Mann-Whitney's non-paired test.
[a]$P < 0.05$ versus placebo; [b]$P < 0.01$ versus placebo

Table 3. Mechanical properties of the opposite (left), non-fractured tibia after local injection of TGF-ß1 around the fracture line of the right tibia. Mean, *SEM*

Experimental groups	n	Ultimate load (N)	Ultimate stress (N/mm^2)	Ultimate stiffness (N/mm)	Young's modulus 10^2N/mm^2	Energy absorption at ultimate load (N × mm)	Deflection at ultimate load (mm)
Placebo	13	80 *2*	238 *4*	230 *8*	112 *3*	27 *2*	0.52 *0.03*
TGF-ß 4 ng/injection	12	83 *2*	235 *5*	255 *8*[a]	115 *4*	25 *2*	0.48 *0.03*
40 ng/injection	10	81 *3*	226 *5*	238 *8*	107 *3*	25 *2*	0.50 *0.03*
P-value (Kruskal-Wallis test)		0.6	0.5	0.04	0.06	0.8	0.4

Mann-Whitney's non-paired test. [a]$P < 0.05$ versus placebo.

Results

The highest dose of TGF-ß (40 ng/injection) resulted in an increase in ultimate load (85 percent compared with placebo, and 75 percent compared with the low dose of TGF-ß), whereas no differences were seen between the groups injected with 4 ng TGF-ß and placebo (Table 1). Ultimate stress, ultimate stiffness, energy absorption at ultimate load, and deflection at ultimate load showed no differences between the groups. Both doses of TGF-ß increased the callus-bone area, whereas no differences were found in the medullary area (Table 2). TGF-ß did not seem to affect the mechanical strength of the opposite intact tibia bone, apart from a 10 percent increase in ultimate stiffness in the group given the lowest dose of TGF-ß (Table 3).

Discussion

In this study, injection of TGF-ß around the fracture line increased the cross-sectional area of callus-bone. Joyce et al. (1990a) showed that daily injections of TGF-ß1 or ß2 in a dose of 20 ng or 200 ng into the

subperiosteal region of newborn rat femur diaphysis resulted in localized bone formation and chondrogenesis. They reported that the cartilage/bone formation ratio was about 3.5 after injection with the highest dose of TGF-ß1, whereas the ratio was zero when the 20 ng dose was used. Their study has now been extended to animals of different ages and has revealed that the degree of response varies with age, being maximal in 6-week-old rats and minimal in aged rats (Roberts and Sporn 1992). Joyce et al. (1990a) dissolved TGF-ß in phosphate buffered saline and no protein carrier like rat albumin was added to protect TGF-ß from sticking to the wall of the tubes and syringes. In our study we prevented TGF-ß from sticking to the walls by adding rat serum albumin to all solutions. We used lower doses of TGF-ß, as Joyce et al. (1990a) found that the ratio of cartilage to bone increased with increasing doses of TGF-ß.

Local application of TGF-ß adjacent to periosteum of the skull increases bone thickness in newborn rats (Noda and Camilliere 1989) as well as in adult mice (Mackie and Trechsel 1990, Marcelli et al. 1990). Using adult rabbits, Beck et al. (1991) found that a single application of TGF-ß1 to skull defects induced a dose-dependent increase in bone formation, when the range of doses were 0.1–2 µg per defect.

Our investigation is the first one dealing with mechanical strength of the healing fracture and TGF-ß treatment. We find that TGF-ß in a dose of 40 ng increases the maximum load, whereas no significant effect is found on the ultimate stress, ultimate stiffness and energy absorption. The absolute values, however, show an increase in ultimate stress of 66 percent, ultimate stiffness of 63 percent and energy absorption of 90 percent, compared with the values of the placebo group. The dispersion in these parameters is considerable in comparison with the one for ultimate load, and this can be the reason why these parameters show no significant differences. TGF-ß in a dose of 4 ng does not seem to influence the mechanical strength of the healing fracture.

In soft connective tissue, it has been found that local application of TGF-ß accelerates mechanical strength development in skin wounds of normal rats (Mustoe et al. 1987, Broadley et al. 1988, Beck et al. 1990); the latter authors showed that application of a single dose of TFG-ß1 on the day of operation enhanced the mechanical strength of the skin wound, and the increase in strength was almost the same when doses in the range of 250 ng to 2500 ng were used, whereas no effect was found when a dose of 25 ng was applied.

Miller et al. (1992) have recently shown that systemic administration of TGF-ß2 increases cancellous bone formation in juvenile and adult rats. However, the dose of TGF-ß2 shown by Miller et al. to possess systemic effects was 5 mg per day for 5 or 14 days, and this is far above the doses we used. Except for a 10 percent increase in ultimate stiffness in the animals given 4 ng, we found no changes in the mechanical strength of intact cortical bone of tibia and, consequently, no systemic effect of 40 ng TGF-ß injected every second day.

Acknowledgements

This work has been supported by Nordic Insulin Foundation and Novos Fonds Komite.

References

Andreassen T T, Seyer Hansen K, Oxlund H. Biomechanical changes in connective tissues induced by experimental diabetes. Acta Endocrinol (Copenh) 1981; 98 (3): 432–6.

Assoian R K. Purification of type-beta transforming growth factor from human platelets. Methods Enzymol 1987; 146: 153–63.

Bak B, Andreassen T T. Reduced energy absorption of healed fracture in the rat. Acta Orthop Scand 1988; 59 (5): 548–51.

Bak B, Andreassen T T. The effect of aging on fracture-healing in the rat. Calcif Tissue Int 1989; 45 (5): 292–7.

Bak B, Andreassen T T. The effect of growth hormone on fracture healing in old rats. Bone 1991; 12 (3): 151–4.

Bak B, Jørgensen P H, Andreassen T T. The stimulating effect of growth hormone on fracture healing is dependent on onset and duration of administration. Clin Orthop 1991; 264: 295–301.

Beck L S, Chen T L, Mikalauski P, Ammann A J. Recombinant human transforming growth factor-beta 1 (rhTGF-beta 1) enhances healing and strength of granulation skin wounds. Growth Factors 1990; 3 (4): 267–75.

Beck L S, Deguzman L, Lee W P, Xu Y, McFatridge L A, Gillett N A, Amento E P. Rapid publication. TGF-beta 1 induces bone closure of skull defects. J Bone Miner Res 1991; 6 (11): 1257–65.

Broadley K N, Aquino A M, Hicks B, Ditesheim J A, McGee G S, Demetriou A A, Woodward S C, Davidson J M. Growth factors bFGF and TGB beta accelerate the rate of wound repair in normal and in diabetic rats. Int J Tissue React 1988; 10 (6): 345–53.

Joyce M E, Roberts A B, Sporn M B, Bolander M E. Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. J Cell Biol 1990a; 110 (6): 2195–207.

Joyce M E, Jingushi S, Bolander M E. Transforming growth factor-beta in the regulation of fracture repair. Orthop Clin North Am 1990b; 21 (1): 199–209.

Joyce M E, Terek R M, Jingushi S, Bolander M E. Role of transforming growth factor-beta in fracture repair. Ann N Y Acad Sci 1990c; 593: 107–23.

Joyce M E, Jingushi S, Scully S P, Bolander M E. Role of growth factors in fracture healing. Prog Clin Biol Res 1991; 365: 391–416.

Kenedi R M. A textbook of biomedical engineering. Blakie and Son, Glasgow 1980: 39–73.

Mackie E J, Trechsel U. Stimulation of bone formation in vivo by transforming growth factor-beta: remodeling of woven bone and lack of inhibition by indomethacin. Bone 1990; 11 (4): 295–300.

Marcelli C, Yates A J, Mundy G R. In vivo effects of human recombinant transforming growth factor-beta on bone turnover in normal mice. J Bone Miner Res 1990; 5 (10): 1087–96.

Miller S C, Rosen D, Armstrong R, DeLeon D, Thompson A, Bentz H, Matthews M, Adams S. Systemic administration of transforming growth factor-beta-2 (TGF-B2) stimulates bone formation in juvenile and adult rats. Abstr 2nd Workshop, Using live Rat in Skeletal Stud, Chicago 1992: 45.

Mustoe T A, Pierce G F, Thomason A, Gramates P, Sporn M B, Deuel T F. Accelerated healing of incisional wounds in rats induced by transforming growth factor-beta. Science 1987; 237 (4820): 1333–6.

Noda M, Camilliere J J. In vivo stimulation of bone formation by transforming growth factor-beta. Endocrinology 1989; 124 (6): 2991–4.

Roberts A B, Sporn M B. Differential expression of the TGF-beta isoforms in embryogenesis suggests specific roles in developing and adult tissues. Mol Reprod Dev 1992; 32 (2): 91–8.