# Periosteal insulin-like growth factor I and bone formation

## Changes during tibial lengthening in rabbits

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**We investigated changes in periosteal insulin-like growth factor I (IGF-I) during tibial lengthening. In 37 rabbits, an osteotomy of the right middle tibia was made and fixed by a unilateral external fixator. The rabbits were randomized into 6 groups: the tibiae were distracted at 0.5 mm/day up to 4 weeks and the animals killed after 2 weeks, 4 weeks or 6 weeks, for each period there was a control group with no distraction. Periosteal IGF-I was measured by radioimmunoassay and bone formation was quantified by CT scanning. During bone lengthening, CT showed moderate bone formation, while IGF-I was increased. When lengthening was stopped, IGF-I returned to a basal level, and CT scanning showed considerable bone formation. Our study suggests that IGF-I plays a role in an early stage of bone formation.**

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Insulin-like growth factor I (IGF-I), a partially growth hormone-dependent polypeptide, has been found in various tissues, and many different tissues are able to synthesize IGF-I (Froesch et al. 1985). Osteoblasts are capable of secreting IGF-I, and in vitro, IGF-I has been found to increase proliferation and protein synthesis in human osteoblasts (Wergedal et al. 1990). There are, however, only a few studies elucidating the role of IGF-I in vivo in bone. Edwall et al. (1992) measured the IGF-I concentration in callus after tibial fractures in rats and found increased levels of IGF-I, suggesting that IGF-I may play a role in the cellular regulation during fracture-healing. Bourque et al. (1993) visualized IGF-I in chondroblasts during fracture repair. Thaller et al. (1993) attempted to accelerate fracture healing of a midfacial bone defect in rats by applying IGF-I directly into the bone defect, and concluded that IGF-I appears to potentiate bone repair. Further, Ammann et al. (1993) reported that local treatment with IGF-I to osteopenic rat tibiae increased the bone mineral density. Mueller et al. (1994) observed stimulation of bone formation and increased trabecular bone volume in adult oophorectomized rats treated with IGF-I. On the other hand, Aspenberg et al. (1989) and Kirkeby and Ekeland (1992) found no stimulatory effect of IGF-I on bone repair.

During bone lengthening, there is a substantial stimulation of bone formation, resulting in a geometrically relatively uniform area of bone. This makes the lengthened bone a good model for studying the process of bone formation. To investigate a possible involvement of IGF-I on bone formation, we measured IGF-I in the periosteal tissue and quantified the bone formation by means of CT during leg lengthening in rabbits.

## Animals and methods

In 37 New Zealand growing rabbits, 5–6 months old, weighing 3–4.5 kg, an osteotomy was made at the middle of the right tibia, just below the junction between the tibia and the fibula. The operations were done under hypnorm/stesolid intramuscular anesthesia and under antibiotic cover (ampicillin 0.5 g intramuscularly). A unilateral dynamic external fixation device (Orthofix M-100) was fixed to the medial side of the right leg, with 2 screws above and 2 screws below the osteotomy. After a latency period of 4 days, the rabbits were randomized into 6 groups. Group 1 was distracted 0.5 mm/day and killed after 2 weeks' distraction (7 mm). Group 2 was distracted 0.5 mm/day and killed after 4 weeks' distraction (14 mm).

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Group 3 was distracted 0.5 mm/day for 4 weeks (14 mm) and killed after 6 weeks. Groups 4, 5 and 6 served as control groups; they were osteotomized and treated in the same way as groups 1, 2 and 3, but no distraction was performed.

The animals were killed with an intracardiac overdose of barbiturate. The distractor was removed, and both legs were stripped of soft tissue, leaving the lengthened area with periost. For IGF-I measurements, parts of the periosteal sheath were taken from the lengthened zone in the distracted groups, from the osteotomy in the osteotomized control group and from corresponding parts of the intact left legs. In addition, serum was obtained from each animal for IGF-I determination.

### IGF-I

IGF-I extraction was performed according to Flyvbjerg et al. (1989). The tissue was homogenized on ice in 1 mol/L acetic acid (5 mL/g tissue) with an Ultra Turrax TD 25 and further disrupted using a potter Elvehjelm homogenizer. The tissue was extracted twice and, after lyophilization, the samples were redissolved in 40 mmol/L phosphate buffer (pH 8.0). Tissue extracts were kept at –80 °C until the assay was performed on diluted extracts. IGF-I was estimated with radioimmunoassay (Flyvbjerg et al. 1989), using a polyclonal IGF-I antibody (Nicols Institute Diagnostics, San Capistiano, CA, USA). For determination of the standard curve and iodination, a full amino acid sequence IGF-I analogue (Amgen Biologicals, CA, USA) was used. Serum IGF-I was measured after extraction in methanol/acetic acid. A linear relationship was found between biosynthetic IGF-I and IGF-I immunoreactivity in rabbit serum or tissue extracts at multiple concentrations, indicating antigen similarity and that no binding proteins or receptors from serum or tissue extracts interfered with the radioimmunoassay.

### CT scanning

CT was performed in consecutive 1.0 mm slices perpendicular to the long axis of the lengthened tibia. The most distal of the proximal screws proved a convenient starting point. Thus 5–13 cross-sectional scans were made across the zone of distraction of the lengthened tibia. The scanning parameters were 2 seconds with 170 mAs at 125 kV. The X-ray absorption (in Hounsfield units [HU] per pixel) was used as a measure of bone density. The CT scanner could provide the area of tissue within certain limits of density (Hounsfield units). To measure the area of mineralized bone, 500 HU was used as the lower limit of mineralization. This value was arrived at by measuring the various tissues in the rabbit. Below 500 HU, there will be some mineralization, but above this value only mineralized tissue is measured. The lengthened area was scanned perpendicular to the axis and divided into zones. Each zone represented the average values of 2 adjacent scans. In the group distracted for 2 weeks, the lengthened area was divided into 3 zones: the proximal, the middle and the distal zone. In the groups distracted for 4 weeks, the data were divided into 5 zones: the most proximal, the proximal, the middle, the distal and the most distal zones.

The tibiae were examined with standardized radiographs having known magnification in anteroposterior and lateral projections. From the CT scanning and from the radiographs, the distracted lengths and the total lengths (from tibial condyles to the ankle) of the tibiae were calculated.

### Statistics

The logarithmic transformed IGF-I data appeared to be normally distributed on a probability plot. Differences in periosteal IGF-I between legs in the same rabbit were analyzed, using the paired t-test and differences in periosteal IGF-I between rabbits were analyzed using the unpaired t-test. One-way analysis of variance was used to test for differences in IGF-I between the groups of serum and between the groups of intact left legs.

The CT data were not normally distributed and differences among groups were evaluated with the Kruskal-Wallis test. The Mann-Whitney two-sample test was used to analyze differences between 2 groups. P-values $\leq 0.05$ were considered significant.

## Results

4 rabbits were excluded from the study: 3 rabbits (groups 2, 4 and 6) suffered from a fracture in one of the drill holes and 1 rabbit (group 6) died from an unknown cause. After 14 days of distraction, the tibiae in the group distracted for 2 weeks were lengthened 6.5 (6.0–7.0) mm (median value (25/75 percentiles)). The tibiae in the group distracted for 4 weeks were lengthened 10.5 (9.0–12.8) mm, and the tibiae in the group distracted for 4 weeks plus 2 weeks' rest were lengthened 12.0 (11.3–12.8) mm. Tibial lengthenings were 6%, 10% and 11% in the 3 groups, respectively.

### IGF-I (Tables 1–3)

The measurements of IGF-I in the serum and in the periosteum of the intact left legs showed no significant differences between the 6 groups. In the distracted groups, the periosteal IGF was increased after 2

**Table 1. Insulin-like growth factor I concentration in periosteal tissue and serum in the distracted legs. Mean value** ***SEM***

| Group | Distracted right leg (ng/g) | Nonoperated left leg (ng/g) | Difference right–left leg (ng/g) | Serum (ng/mL) |
|---|---|---|---|---|
| 2 weeks (n 6) | 80* *24* | 50 *12* | 30 *15* | 612 *73* |
| 4 weeks (n 6) | 122** *21* | 67 *7* | 54 *22* | 625 *78* |
| 4+2 weeks rest (n 6) | 58 *8* | 54 *7* | 4 *8* | 596 *92* |

* p 0.05 compared with the nonoperated left leg, ** p 0.07 compared with the nonoperated left leg.

**Table 2. Insulin-like growth factor I concentration in periosteal tissue and serum in the distracted legs versus the osteotomized control legs. Mean value** ***SEM***

| Group | Distracted leg (ng/g) | Osteotomized control leg (ng/g) |
|---|---|---|
| 2 weeks | 80 *24* | 85 *19* |
| 4 weeks | 122* *21* | 67 *12* |
| 4+2 weeks | 58 *8* | 65 *7* |

* p 0.05 compared with the osteotomized control legs.

**Table 3. Insulin-like growth factor I concentration in periosteal tissue and serum in the osteotomized control legs. Mean value** ***SEM***

| Group | Operated right leg (ng/g) | Nonoperated left leg (ng/g) | Difference right–left leg (ng/g) | Serum (ng/mL) |
|---|---|---|---|---|
| 2 weeks (n 5) | 85 *19** | 37 *4* | 48 *15* | 462 *22* |
| 4 weeks (n 6) | 67 *12* | 59 *6* | 8 *11* | 553 *24* |
| 6 weeks (n 4) | 65 *7* | 64 *13* | 2 *7* | 615 *28* |

* p 0.006 compared with the nonoperated left leg

**Table 4. Area of mineralized bone (HU > 500) in the lengthened zone. Median values** ***25–75 percentiles***

| Groups | Most prox. | Prox. | Middle | Distal | Most dist. |
|---|---|---|---|---|---|
| 2 weeks (n 6) | | 18 *7–21* | 10 *3–11* | 13 *6–19* | |
| 4 weeks (n 6) | 32 *21–51* | 32 *27–33* | 10 *9–17* | 19 *12–23* | 42 *33–45* |
| 4+2 weeks (n 6) | 56 *37–74* | 63* *51–74* | 58* *46–63* | 58* *46–76* | 69 *40–83* |

*p < 0.05 compared with 2- and 4-week groups.

weeks' distraction, compared to the paired left legs (p 0.05). After 4 weeks' distraction, an increase in periosteal IGF-I was found when compared to the paired left legs (p 0.07) and when compared to the control group (p 0.05). After 4 weeks' distraction followed by 2 weeks' rest, no differences in periosteal IGF-I were found between the distracted and the paired left legs or the control group. In the control groups, the periosteal IGF-I was increased after 2 weeks, compared to the paired left legs (p 0.006). After 4 and 6 weeks no differences were noted compared to the paired left legs.

### *CT scanning (Table 4)*

CT measurements of bone area in the groups distracted for 2 and 4 weeks showed a decrease of bone towards the middle during lengthening. In the group rested for 2 weeks after lengthening had stopped,

there was an increase in the area of bone in the proximal (p 0.004), middle (p 0.004) and distal zones (p 0.004), compared to the group killed after 4 weeks' distraction. Although there was a difference in the area of bone in the most proximal zone and in the most distal zone between the group distracted for 4 weeks and the group distracted for 4 weeks, followed by 2 weeks' rest, this was not significant.

## Discussion

We found only moderate amounts of mineralized bone with a decrease in bone towards the middle of the lengthened zone during lengthening. The decrease in mineralized bone towards the middle of the lengthened zone confirms findings by Aronson et al. (1990) who measured bone density during bone lengthening by CT scanning. They divided the lengthened bone into 5 distinct zones and found a decrease in density towards the middle zone. Van Roermund et al. (1987, 1991) studied bone formation after distraction epiphysiolysis in rabbits using CT, but they concentrated on the period after distraction. They observed a decrease in the density towards the middle in the lengthened epiphysis, even 18 weeks after lengthening had stopped. This is in contrast to our findings where considerable bone formation was observed in the whole lengthened zone 2 weeks after lengthening had stopped. A difference in the localization of lengthening or a difference in fixation rigidity may explain this discrepancy. We found that CT measurements of mineralized bone callus dividing the lengthened area into distinct zones provide a noninvasive quantitative evaluation of the formation of new bone in lengthened bone.

We observed an early and pronounced increase in periosteal IGF-I during the phase of lengthening when CT scanning showed only small areas of mineralized bone. When lengthening stopped, the concentration of IGF-I returned to basal level and CT showed considerable bone mineralization. However, there is still a question of cause and effect of the increase in concentration of IGF-I, but, taken together with the known in vitro effects of IGF-I (Wergedal et al. 1990), the findings indicate that IGF-I may stimulate the osteoblasts to bring about the early proliferative phase of bone formation.

In line with the possible stimulatory effect of IGF-I during bone lengthening, our study also provides evidence for a stimulatory effect of IGF-I during bone healing after osteotomy. An early and transient increase in periosteal IGF-I observed in the osteotomized tibiae after 2 weeks, returned to basal levels after 4 and 6 weeks. This agrees well with the results obtained by Edwall et al. (1992) in a rat tibia fracture model. They found increased IGF-I in callus, with a peak value 1 week after fracture.

The increase in periosteal IGF-I could be due either to increased uptake from the blood or to local production of periosteal IGF-I. Increased uptake from the circulation could occur through increased binding of IGF-I to specific IGF-I receptors or binding to one of the 6 known IGF binding proteins. Nevertheless, we found no significant differences in serum IGF-I between the 6 groups of rabbits. Edwall et al. (1992) administered indomethacin to a rat tibia fracture resulting in decreased IGF-I mRNA expression. Indomethacin is known to inhibit prostaglandin synthesis and thereby the inflammation. The findings by Edwall et al. suggest that activation of IGF-I during tissue regeneration may be mediated either by prostaglandin or by the inflammatory response to the trauma. The hypothesis of prostaglandin as the local activator of IGF-I is supported by Keller et al. (1993) who found increased IGF-I in periosteal tissue after local PGE-2 infusion at a plated tibial osteotomy in rabbits.

Our findings support a role for IGF-I as a stimulator at an early stage in bone formation. The cellular mechanisms may operate through a complex system comprising changes in IGFs, IGF receptors and IGF binding proteins. Further studies measuring these parameters are necessary for defining the exact role of IGF during bone formation.

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