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Growth factors in distraction osteogenesis

Immuno-histological pattern of TGF- β 1 and IGF-I in human callus induced by distraction osteogenesis

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Abstract Although growth factors have been demonstrated during bone healing, their presence has not yet been confirmed in callus distraction. Therefore, in 3 patients we searched for cytokines during callus distraction. Bone biopsies were immuno-histochemically stained for TGF- β 1, IGF-I, TGF- β type II receptor, IGF receptor, and proliferating cell nuclear antigen (PCNA). Histologically we found immature woven bone in the middle of the callus zone and increasing calcification and lamellar bone in the re-modelling zone. Osteoblasts and fibroblast-like cells in the middle zone, and osteoblasts in all zones stained for TGF- β and its receptor. The number of positive staining cells related to proliferous activity as assessed both by PCNA, and by bone density in radiographs. IGF-I could be detected everywhere. In conclusion, growth factors are present in bone formation and in areas of re-modelling during callotaxis. Their relation to proliferous activity and radiographic density supports their involvement in osteogenesis.

Résumé La présence de facteurs de croissance a été montrée dans la consolidation osseuse mais pas encore établie dans les cals par distraction. Nous avons étudié les cytokines durant la distraction chez 3 patients. Des examens immuno-histo-chimiques ont été faits à partir de biopsie pour étudier les facteurs TGF- β , IGF-I, récepteurs TGF- β II, récepteurs IGF et l'antigène nucléaire de prolifération (PCNA). Histologiquement, nous avons trouvé de l'os immature dans la zone médiane du cal et, dans la zone de remodelage une calcification avec os lamellaire. Les ostéoblastes en général et les cellules fibroblastes-like de la zone médiane ont été positives pour le facteur TGF- β et son

récepteur. Il existe une corrélation entre la quantité de cellules positives et l'activité proliférative trouvée par la PCNA et par la densité radiologique. Le facteur IGF-I a été détecté ubiquitairement. En conclusion, les facteurs de croissance sont présents dans l'os en formation et en remodelage des cals par extension. La corrélation entre l'activité proliférative cellulaire et la densité radiographique, souligne leur rôle dans le processus ostéogénique.

Introduction

The interaction of various growth factors during bone healing and re-modelling has been studied extensively in recent years, mostly in animal models [4–6, 10], but clinical studies are limited. Transforming growth factor beta 1 (TGF- β 1), insulin-like growth factors I and II (IGF-I and IGF-II) and platelet-derived growth factor (PDGF) have been recently demonstrated during normal human fracture healing [1–3].

Clinically, rapid bone formation can be produced by distraction osteogenesis [13–15, 24] and there is some evidence of the presence of growth factors which results in new bone [11]. However, the occurrence and distribution of cytokines within the area of bone formation have not previously been established in humans. We therefore investigated the patterns of TGF- β 1 and IGF-I in the distraction callus of the femur and tibia in 3 patients.

Materials and methods

During necessary revision surgery, bone specimens were obtained from 3 patients undergoing callus distraction. All the patients gave informed consent for the biopsy, and Institutional Review Board Approval was obtained for this study. The specimens were examined using histological and immuno-histochemical techniques.

Case reports

Case 1

A 22-year-old male had been treated in Croatia for a femoral shaft fracture by open reduction and plating. A deep infection led to os-

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teitis with death of bone and conventional treatment was unsuccessful. In our institution, resection of the necrotic mid-diaphysal section of the femur was performed and this was followed by callus distraction and segment transport (1 mm/day) using an AO external fixator [22]. Premature consolidation of the distracted callus after 43 days prevented adequate transport and led to revision surgery. The distal part of the distraction area was osteotomised and transport was resumed after 7 days using a faster rate (1.8 mm/day). When transport and docking had been completed at 94 days, fixation was performed with a condylar plate. Bone specimens were taken from the callus during both slow (1 mm/day) and fast (1.8 mm/day) distraction.

Case 2

A 29-year-old male with a grade III A open tibial fracture was treated primarily by external fixation and then several days later by secondary intramedullary nailing. Deep infection and osteonecrosis occurred, and after admission to our hospital local myoplasties and extensive autogenous bone grafting failed to heal the infected non-union. Segmental resection was performed and this was followed by proximal tibial corticotomy and the start of callotaxis. As callus formation was poor, the transportation rate was reduced to 0.6 mm/day. Several further operations were necessary due to the recurrence of local infection. Specimens of the distraction zone were taken during the docking operation at 199 days. Calcification of the distraction callus was slow and osseous consolidation eventually occurred.

Case 3

A 19-year-old female presented with chronic destructive osteitis of the distal femur. Diagnosis was confirmed by radiography, CT, MRI and histology. The distal part of the femur, the knee joint and the proximal end of the tibia were resected. This was followed by proximal tibial and femoral corticotomy, and bifocal callus distraction with the transportation segments being moved toward each other. After the re-appearance of a local swelling, investigation revealed a primary B-cell-lymphoma of the distal femur. Severe local necrosis with super-infection and generalised sepsis necessitated a thigh amputation 93 days after the start of callus distraction. Specimens of the distraction callus were obtained at this time.

Histology and immuno-histochemical preparation

Bone biopsies were placed in 4% buffered paraformaldehyde solution for 48 h. They were then decalcified in 20% EDTA solution. When fixed, the tissue was cut into 2 mm serial sections, and these were deparaffinised and stained with hematoxylin for further immuno-histochemical investigation. Sections for histological examination were stained according to van Kossa and Goldner.

The presence of TGF- β 1 and TGF- β type II receptors in the biopsies was detected with TGF- β 1 rabbit antibody (Santa Cruz Biotechnology Inc. CA, USA) and a TGF- β type II receptor rabbit antibody (Santa Cruz Biotechnology Inc., CA, USA). IGF-I and IGF-II receptors were also detected by the use of rabbit antibodies (Santa Cruz Biotechnology Inc., CA, USA). Cell proliferation was revealed by using primary cell nuclear antigen (PCNA) prepared from mice (Oncogene Science, Uniondale, NY, USA) [8].

TGF- β 1, TGF- β receptor type II and PCNA immuno-reactivity were demonstrated by using the avidin-biotin-complex (ABC) method with 3,3'-diaminobenzidine (DAB) serving as chromogen [12]. In brief, tissue sections were incubated overnight at 4°C with TGF- β 1 antibody (1:100), TGF- β type II receptor antibody (1:100) or PCNA antibody (1:75) followed by biotinylated swine anti-rabbit IgG (Dako, Fluka, Buchs, Switzerland) or biotinylated rabbit anti-mouse IgG (Dako, A/S, Denmark) for 30 min. Finally, sections were incubated with ABC and 0.05% DAB/0.003% hy-

drogen peroxide (Fluka, Buchs, Switzerland). Negative control slides were incubated with PBS (phosphate-buffered saline solution).

Staining was classified as non, +, ++ or +++, depending on the intensity.

Results

Clinically, varying degrees of osteogenesis could be induced in all cases by callus distraction. In Case 1, slow distraction led to premature ossification, and this required a second corticotomy in order to allow segmental transportation to continue. A fast distraction rate produced a moderate amount of callus formation (Fig. 1a). In Case 2, in which the infection recurred, callus formation was poor (Fig. 1b), but osseous consolidation was finally achieved. In Case 3, no problems resulted from callus distraction (Fig. 1c).

Histologically, varying amounts of bone formation were observed. While woven bone and lamellar bone could be identified in different areas of the newly formed bone in Case 1 and Case 3, fibrous tissue with only rare bone trabeculae could be found in Case 2. These findings correlated well with the radiographic appearance of the distraction callus. In the central distraction zone of Case 3, where the "youngest" distraction callus was found, a few irregularly shaped osseous structures with little calcification could be seen (Figs. 2a, 2b). In later distraction callus the bone formed was similar to regular lamellar bone and showed widespread calcification (Figs. 2c, 2d).

PCNA-stained cells could be found in osteoblasts adjacent to the newly formed bone trabecula. Fibrous tissue contained fibroblasts with PCNA positive nuclei (Fig. 3a). The relative quantity of PCNA positive cells showed a good correlation to the radiographic appearance of the callus (Table 1). In Case 2, in which failure of the distraction osteogenesis was suspect at an early stage, only a few cell nuclei stained positively for PCNA. In Case 3, in which specimens from the active middle zone and the border zone could be compared, cells in the middle zone stained much more positively for PCNA than in the zone adjacent to normal bone where the osteogenic activity is lower and the newly formed bone is older (Fig. 3b).

Cells staining positive for TGF- β 1 could be found in every region of the distraction callus, mostly in osteo-

Fig. 1a–c Plain radiographs of the distraction area at the time of biopsy. **a** Case 1: Callotaxis of the right femur: note the densities of the distraction callus resulting from 2 different distraction rates. **b** Case 2: Callotaxis of the tibia. Impaired bone formation after 190 days of distraction. **c** Case 3: Callotaxis of the femur. Regular bone formation after 90 days of distraction

Fig. 2a–d Histology of the distraction callus. **a** Middle area of the distracted zone. Vivid osteogenesis, immature bone structures with irregular trabeculae and fibrous tissue (Masson-Goldner, bar 50 μ m). **b** Middle area of distraction zone. Early calcification (von Kossa, bar 50 μ m). **c** Re-modelling zone adjacent to corticotomy site, lamellar bone structures, and less fibrous tissue surrounding trabecula (Masson-Goldner, bar 300 μ m). **d** Extended calcification in proximal re-modelling zone (von Kossa, bar 300 μ m)

Table 1 Radiographic appearance, histology and immunohistochemical results at different biopsy sites during callotaxis. +, ++ and +++ represent relative amount of cells staining positive

Case	Specimen	Biopsy site	Radiographic callus density	Histology	PCNA	TGF	TGF-R	IGF	IGF-R
1	I	Distraction site, "slow" transportation rate	High	many trabecula, osteoblasts	+++	+++	+++	+++	+++
	II	Distraction site, "fast" transportation rate	Medium	Many trabecula, osteoblasts, fibroblast like cells	+++	+++	+++	+++	+
2	I	Distraction site, poor bone formation	Low	Rare bone trabecula, fibrous tissue with rare fibroblasts	+	+	+	0	+
	II	Distraction site, poor bone formation	Low	No bone trabecula, fibrous tissue, fibroblasts	+	+	++	+	+
3	I	Distraction site, adjacent to normal bone (proximal)	High	Cancellous bone, Low osteoblastic activity	+	+	+	++	+
	II	Distraction site, Highly osteogenetic middle zone	Medium	Trabecula, osteoblastic activity, fibroblasts	+++	++	++	++	+
	III	Distraction site, adjacent to normal bone (distal)	High	Ripe cancellous bone, Low osteoblastic activity	+	++	++	++	+

blasts on the surface of bone trabeculae (Fig. 4a). The amount of TGF- β , however, was found to be higher in the central distraction zone with immature bone structures, and lower in the zones adjacent to the osteotomy site (Fig. 4b). TGF- β positive cells were rare in Case 2, and this was associated with impaired callus formation (Fig. 4c). Receptors for TGF- β demonstrated exactly the same pattern of distribution as TGF- β (Fig. 4d). Overall, the activity of TGF- β was most pronounced in those regions of the distraction callus where rapid cell proliferation was taking place. This was assessed radiographically and by staining for PCNA (Table 1).

IGF-I could be found nearly everywhere in every region of distraction callus (Fig. 5a). The specimen in Case 2 showed only weak staining for IGF-I. Receptors for IGF-I, on the other hand, were limited (Fig. 5b). Only one specimen in Case 1 ("slow distraction") stained positive for IGF-I receptors.

Discussion

Chemotaxis, cellular differentiation and synthesis of extra-cellular matrix are important during fracture healing. This biological process is known to be highly regulated and in different regions we find the same time-dependent re-modelling pattern [5]. Bone formation during distraction osteogenesis is not only a time-dependent but also a zone-dependent process. Under optimal conditions a thin osteogenic layer resembling a growth plate-like structure can be demonstrated histologically in the middle of the distraction space which Ilizarov called "the growth-zone of the distraction-regenerated region" [13, 15]. In an animal model, clusters of depositing osteoblasts that accounted for the longitudinal bone growth could be observed at the free end of bone trabeculae near the distraction gap [9]. In our biopsies we confirmed the presence of the immature osseous structures in the middle zone of callus distraction. In Case 3, where bone specimens of different areas were examined, the specimens adjacent to the corticotomy and adjacent to intact periosteum demonstrated mature osseous structures. The re-modelling process with formation of lamellar bone and calcification was already occurring at the time of biopsy.

Proliferating cell nuclear antigen has been recently studied for assessment of cell proliferation during experimental bone lengthening and a correlation with the distraction rate was found [16]. In our study the number of proliferating cells detected by immuno-histochemical staining for PCNA correlated well with the density of newly formed bone as assessed radiographically.

TGF- β is known to induce differentiation and proliferation of osteoblasts and also to stimulate the appear-

◀ **Fig. 3a,b** Immuno-histology, staining for PCNA. **a** Demonstration of proliferating activity in osteoblasts on the surface of newly-formed trabeculae. Note proliferous activity in the fibroblasts in adjacent fibrous tissue (bar 30 μ m). **b** Less proliferous activity in the proximal re-modelling zone in Case 3 (bar 50 μ m)

Fig. 4a-d Immuno-histology, staining for TGF- β 1 and Type II receptors for TGF- β . **a** Positive osteoblasts (arrows) on the surface of trabeculae (bar 75 μ m). **b** Positive osteoblasts (arrows) in the proximal re-modelling zone of Case 3 (bar 50 μ m) **c** Rare osseous structures with few positive cells (Case 2) with impaired callus formation (bar 75 μ m). **d** Positive staining for receptors for TGF (small arrows), same region as Fig. 4a (bar 120 μ m)

Fig. 5a,b Immuno-histology, staining for IGF-I. **a** Detection of IGF in osteoblasts (bold arrows), osteocytes (open arrows) and fibrocytes (curved arrow) (bar 300 μ m). **b** Weak staining for receptors for IGF-I in same region (bar 185 μ m)

ance of extra-cellular matrix proteins [4, 6]. Fracture healing was enhanced by application of TGF- β in various experimental settings [4, 17, 21]. In an animal fracture model, TGF- β was observed in calcified matrix at the ossification front penetrating the cartilage callus in later stages of fracture repair [7]. These findings suggest an important role for TGF- β in stimulating these processes during bone healing. In this biopsy study we investigated whether TGF- β could also be found in distraction callus. In the distraction zone TGF- β was detected in various cells such as osteoblasts and fibroblasts. The expression of TGF- β was most pronounced in those regions of the distraction callus with rapid cell proliferation as assessed by increased PCNA. Interestingly, biopsies of patients with good bone formation as assessed by radiography demonstrated high proliferous activity and a high level of growth factor. On the other hand, in the patient with impaired bone formation a lack of proliferous activity and growth factors were seen. Therefore we suggest that TGF- β plays an important role not only in bone healing but also in bone formation during callus distraction. The presence of type II receptors for TGF gives further evidence for the action of TGF- β . It has been shown that mechanical stress leads to an increase in growth factor [20]. One might therefore assume that mechanical stress during callus distraction is an additional factor for growth factor secretion. TGF- β is known to be released during callus distraction and was found in the serum of patients undergoing these procedures [11].

IGF-I is known to be an important cytokine for many biological processes and acts through endocrine, paracrine and autocrine pathways [23]. During fracture repair IGF-I can be found in chondroblasts during early callus formation [7]. A number of studies using different model systems have demonstrated that both IGF-I and IGF-II stimulate the proliferation of osteoblasts and also enhance different functions such as the quality and presence of extra-cellular matrix proteins [18]. In human fracture healing mRNA for both IGF-I and IGF-II was found in osteocytes and young chondrocytes during cartilage and bone formation [2]. *In vivo* IGF-I stimulated bone formation and bone healing in various animal models [19, 25]. In our study we visualised IGF-I in fibroblasts and osteoblasts in human distraction callus. The presence and extent of IGF in our biopsies corresponded well to the clinical course. The low receptor density which was found may be due to a down-regulation effect, caused by an increased secretion of IGF-I.

In the present study, TGF- β and IGF-I were detected immuno-histochemically in a distinct area of bone formation. The question whether cells stained for TGF- β and IGF-I could be the source or the target of growth factors can only be answered by the use of *in situ* hybridisation [1].

All cases reported in this study had had a previous infection. The presence of growth factors may be influenced by the infection, although in Case 1 and Case 3 the infection was not present at the time and/or at the site

of biopsy. However, in patients without any infection there was no need for surgical exposure of the distraction site and so no biopsy material could be obtained.

Our results reveal the distribution of 2 growth factors at specific times after initiation of callotaxis. Earlier or later phases in callotaxis might have different patterns of growth factors involved in initiation and re-modelling of bone. The time-dependent patterns of growth factors during callotaxis should be measured quantitatively in further experimental studies by moleculo-biological methods.

In conclusion, our data suggest that TGF- β 1 and IGF-I are present and important during bone formation in callus distraction and that they stimulate cell proliferation and extra-cellular matrix formation.

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