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Expression of four growth factors during fracture repair

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ABSTRACT Fracture repair offers an opportunity to study the physiology of bone formation at the fracture site. Isolation of growth factors from bone matrix has implicated growth factors as participants in bone physiology. We therefore examined the expression patterns of aFGF, IGF-I, PDGF, and TGF-ß during fracture repair. An animal model has been developed to study repair of tibial fractures. The model provides both reproducible and quantifiable results, allowing the fracture repair process to be divided into four stages (Bourque *et al., Lab. Anim. Sci 42:* 369-374, 1992). Fractured tibiae were examined immunohistochemically with polyclonal antibodies to four growth factors. PDGF was visualized in macrophages in close proximity to the periosteum during stage 1. aFGF was visualized in cells of the expanded cambial layer and was associated with a rapid increase in the population of fibroblast-like mesenchymal cells during stage 2. IGF-I was visualized in young chondroblasts at the edge of the cartilage mass replacing the fibrous callus during stage 3. TGF-ß was visualized in calcified matrix producing chondrocytes at the edge of ossification fronts penetrating the cartilage callus during stage 4. The immunohistochemical results suggest that these growth factors act as local simulators of the repair process.

KEY WORDS: *growth factors, fracture, immunohistochemistry, bone*

Introduction

Much published information exists which documents the involvement of growth factors in connective tissue physiology. A great deal of this research involves observations made from *in vitro* experimental systems. *In vivo* studies require the assessment of more variables and consequently, less work has been published in this area. In this study, several growth factors are immunolocalized and their distribution throughout the fracture repair process is described.

In wound healing, the inflammatory phase follows a time course where macrophages appear at the wound site, followed by fibroblasts (Ross and Benditt, 1961). This association led Leibovich and Ross (1975) to propose that macrophages stimulated proliferation of fibroblasts. Subsequent investigations by other researchers demonstrated that macrophages could produce FGF, PDGF, and TGF-ß (Baird *et al.*, 1985; Shimokado *et al.*, 1985; Assoian *et al.*, 1987). Wound fibroblasts are not constrained to remain as fibroblasts but can differentiate into other mesodermal cell types, most notably, chondrocytes (Ahrens *et al.*, 1977). The ability of wound fibroblasts to transform into chondroblasts under appropriate environmental conditions is important during limb regeneration and also during fracture repair.

A major source of osteogenic cells in long bones is the periosteum. It has an outer fibrous layer and an inner osteogenic layer (Ham and Cormack, 1979). Experiments involving removal of this inner layer of osteogenic cells and *in vivo* transplantation demonstrated the ability of these cells to differentiate directly into osteoblasts or chondrocytes (Nakahara *et al.*, 1990). Eyre-Brook (1983) reported that the periosteum is stimulated to form bone when it is surrounded by a hematoma, and confirmed osteogenic potential of the periosteum to be limited to the inner or cambial layer.

Bone matrix acts as a reservoir for many extracellular proteins which include growth factors such as FGF (Hauschka *et al.*, 1986), TGF-ß (Seyedin *et al.*, 1985) and IGF (Frolik *et al.*, 1988). The effect of administration of aFGF, PDGF, and TGF-ß on normal fracture healing has been examined in a rat fracture model (Jingushi *et al.*, 1990; Joyce *et al.*, 1990a,b). All were found to have an effect on the osteogenic response to fracture.

Four distinct and reproducible stages in fracture repair have been described using the CD-1 mouse as an animal model (Bourque *et al.*, 1992). Macrophages are one of the major cell types present during the first stage of fracture repair. Following their appearance, fibroblast-like cells begin to accumulate in and around the fracture site. Toward the latter part of stage 1, cell division can be seen in the cambial layer of the periosteum. PDGF is produced by macrophages and is a potent stimulator of cell division in cells of mesodermal origin such as chondrocytes and fibroblast-like cells

Abbreviations used in this paper: PBS, phosphate buffered saline, pH 7.2; PDGF, platelet derived growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; IGF-I, insulin-like growth factor one; TGF-ß, transforming growth factor beta; BMP, bone morphogenetic protein.

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(Canalis *et al.*, 1989; Wroblewski and Edwell, 1992). For these reasons, PDGF is likely to be involved in fracture repair and more specifically during the early stages.

aFGF is a potent stimulator of cell division in fibroblasts and cells with chondroprogenitor and osteoprogenitor characteristics (Canalis and Raisz, 1980; Kato and Gospodarowicz, 1984; McCarthy *et al.*, 1989). The second stage of fracture repair involves replication of fibroblast-like cells and chondroprogenitor cells in the cambial layer of the periosteum. It was therefore hypothesized that aFGF would be involved.

The third stage of fracture repair involved chondrogenesis, both proliferation of chondrocytes and production of cartilage matrix. IGF-I is a stimulator of both cartilage matrix production and chondrocyte proliferation (Bohme *et al.*, 1992). It was suggested that IGF-I participated in chondrogenesis.

The last stage of fracture repair involves production of bone through endochondral ossification. TGF-ß is a potent stimulator of cell division and matrix production in cells of chondrogenic and osteogenic origin (O'Keefe *et al.*, 1988; Hock *et al.*, 1990). TGF-ß was studied immunohistochemically for a role during the osteogenic stage.

Results

At day 1 post-fracture, many monocytes were present in the fracture site, but few had transformed into macrophages. It was not until day 2 that the proportion of macrophages increased relative to the proportion of their precursor cells, the monocytes. Monocytes appeared to be centered in the fracture site and associated with the hematoma. Macrophages, on the other hand, occurred in increasing numbers away from the fracture site. PDGF was identified during stage 1 of fracture repair. At 2 days post-fracture, many macrophages appeared at the periphery of the fracture site, in close proximity to the periosteum. Antibodies to PDGF applied to tissue sections from this stage of the repair process (day 2 post-fracture) produced positive staining on macrophages (Fig. 1A,B,C,D). In tissue sections from day 3 post-fracture, the number of macrophages staining for PDGF decreased along with the total population of macrophages. The largest number of PDGF-producing macrophages coincided with initiation of cell division in the cambial layer of the periosteum. Other cell types present during this stage included monocytes, fibroblast-like mesenchymal cells, polymorphonuclear leukocytes, bone marrow cells, and osteocytes from pre-existing bone. None of these other cell types proved positive for PDGF.

Staining for aFGF was confined to cells of the expanding cambial layer of the periosteum. Even though cellular activity could be observed in the cambial layer at day 2 post-fracture, reactivity of aFGF was not detected until day 3 post-fracture, coinciding with stage 2 of the repair process (Fig. 2A,B,C,D). From day 3 to day 5 post-fracture, the cambial layer expanded as a result of an increased cell population. There was a difference between the shape of the day 2 and day 3 post-fracture cambial layer cells. The day 2 cells were flattened and fibroblast-like in appearance. The day 3 cambial layer cells became rounded and began producing a cartilaginous matrix (as indicated by light alcian blue staining of the matrix). Beyond day 5 post-fracture, the cambial layer became indistinguishable from the cartilage tissue which was transforming the fracture callus. Other cell types present during the second stage of fracture repair included fibroblast-like mesenchymal cells, macrophages, bone marrow cells, endothelial cells, osteoblasts, and osteocytes from pre-existing bone. None of these other cell types proved positive for aFGF.

During the third stage of fracture repair, cartilage replaced the fibrous fracture callus. This process occurred from day 5 post-fracture to day 9 post-fracture. An essential feature of this process was transformation of fibroblast-like mesenchymal cells into chondroblasts, an event similar to the transformation of wound fibroblasts to chondrocytes during limb regeneration in amphibians (Ahrens *et al.*, 1977). The number of fibroblast-like mesenchymal cells increased from day 3 to day 5 post-fracture at which time cell density appeared to reach a maximum. Starting at day 5 post-fracture, fibroblast-like mesenchymal cells at the center of the cell mass became rounded and started producing a cartilaginous matrix. See Fig. 3B. Cartilage formed and began to spread out, chondrocytes on the inside of the cartilage stained intensely blue indicating the presence of a much greater amount of chondroitin sulfate. Young chondroblasts at the periphery of the cartilage stained light blue. Cartilage was most conspicuous from day 7 to day 9 post-fracture. Young chondroblasts at the edge of the cartilage during this time period stained positively for IGF-I (Fig. 3 A,B,C,D). Other cell types present during this third stage of repair included fibroblast-like mesenchymal cells, endothelial cells, mature chondrocytes, bone marrow cells, and osteocytes from pre-existing bone. None of these stained positively for IGF-I. Also in tests on tissue sections from stages 1, 2 and 4 (days 3-9 post-fracture), containing macrophages, cambial layer cells, and osteoblasts, no staining for IGF-I was observed.

During the fourth stage of fracture repair (days 9 to 20 post-fracture), the cartilage was infiltrated by blood vessels and osteogenic cells and replaced through endochondral ossification. Some transformation of cartilage to bone occurred at the very edge of the cartilage callus as early as day 7 post-fracture, but the process took approximately two days to reach the fracture site. The most conspicuous cell types present during this fourth stage of repair were chondrocytes and osteoblasts. Using direct red (a histochemical stain), calcified matrix stained red. Chondrocytes, at the edge of the ossification front infiltrating the cartilage mass, transformed into calcified matrix-producing cells, signified by the appearance of a red-stained rim within the chondrocyte lacunae. This marked a transition of chondrocyte function from maintaining a proteoglycan matrix to producing a calcified matrix similar to that found in bone.

At this fourth stage, intense staining for TGF-ß occurred in the calcified matrix-producing cells at the edge of the ossification front (Fig. 4A,B,C,D). Other cell types present during this stage of fracture repair were bone marrow cells, osteocytes, and endothelial cells, none of which stained for TGF-ß. Neither did other cell types present during stages 2 and 3 (days 5-9 post-fracture). Table 1 summarizes the immunoreactive cell types for each growth factor.

Discussion

Immunohistochemical studies using commercially available antibodies demonstrated the presence of four growth factors in the tissues in and around the fracture site. Their association with particular cell types and tissue processes during the stages of repair suggested that they play a role as local stimulators of the repair process.

Macrophages play a prominent role during the inflammatory response to injury. Their precursor cells, monocytes, first appear in the hematoma but transform into macrophages within hours of

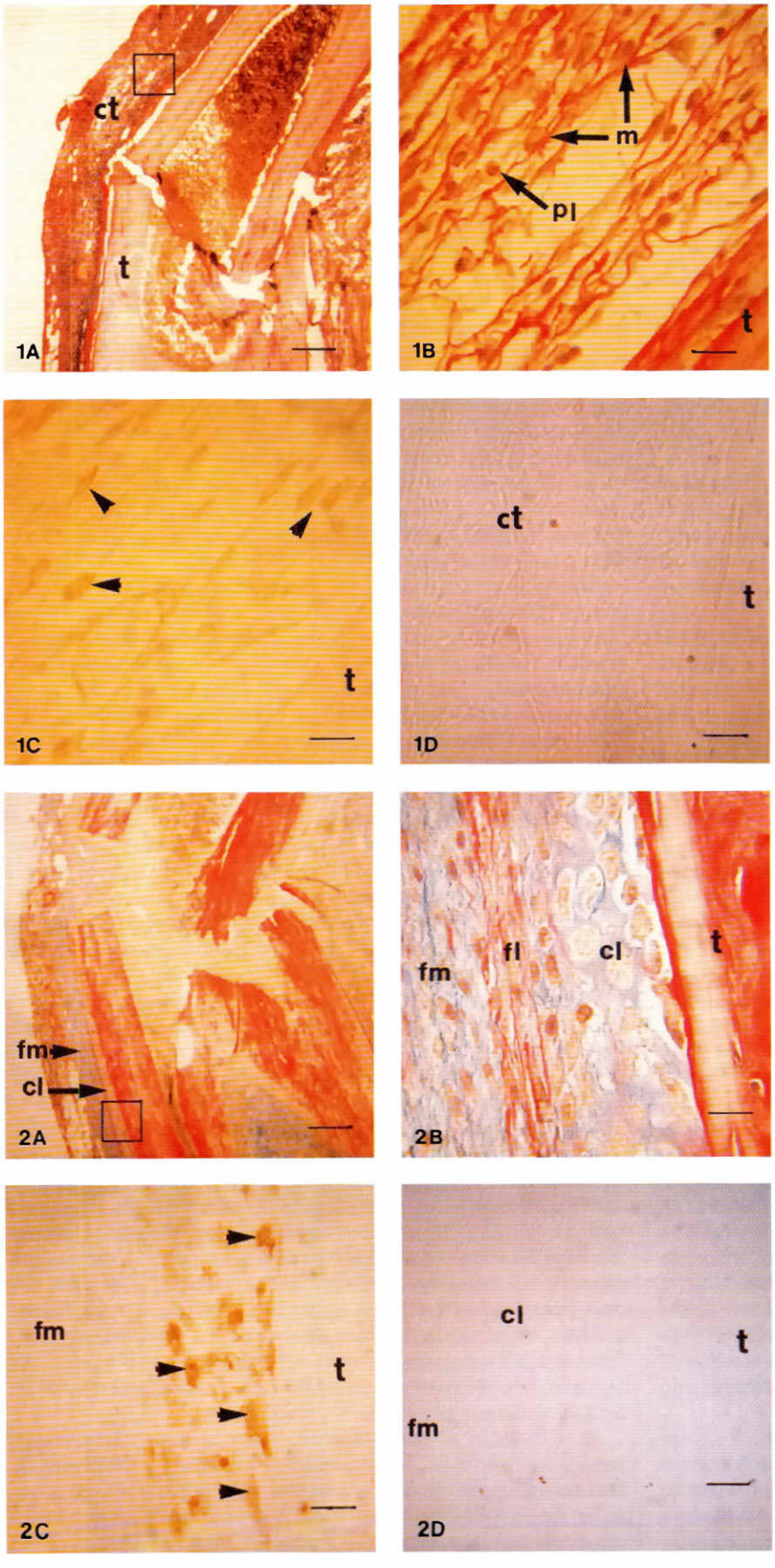

Fig. 1. Immunohistochemical detection of PDGF during stage 1 of fracture repair. (A) *Low-power photomicrograph of histological section from a day-2 post-fracture tibia stained with HBQ method. Note the tibia (t), and loose areolar connective tissue (ct). Bar, 192 μm.* **(B)** *High-power photomicrograph of area in inset from (1A). The tissue type present is mainly areolar connective tissue. Note the many macrophages (m). Also present are polymorphonuclear leukocytes (pl) and the tibia (t). Bar, 21 μm.* **(C)** *Serial adjacent section to one in (1B) with polyclonal antibodies to PDGF applied. Note positive staining only on macrophages (arrow heads) and not tibia (t). Bar, 21 μm.* ***(D)*** *Serial adjacent section to one in (1C) omitting primary PDGF antibody. Note absence of any pigmentation in connective tissue (ct) or tibia (t). Bar, 85 μm.*

Fig. 2. Immunohistochemical detection of aFGF during stage 2 of fracture repair. (A) *A low-power photomicrograph of the fracture site at day-3 post-fracture stained with HBQ method. Note that the cambial layer (cl) is visible as well as an accumulation of fibroblast-like mesenchymal cells (fm). Bar, 192 μm.* **(B)** *A high-power photomicrograph of area in inset from (2A). From right to left, note the tibia (t), cambial layer (cl), outer fibrous layer (fl) of the periosteum, and the accumulation of fibroblast-like mesenchymal cells (fm). Bar, 21 μm.* **(C)** *Serial adjacent section to one in (2B). Note the intense staining of the cambial layer cells with antibodies to aFGF (arrow heads), and absence of staining in pre-existing osteocytes of the tibia (t) and fibroblast-like mesenchymal cells (fm). Bar, 21 μm.* **(D)** *Serial adjacent section to one in (2C) omitting primary aFGF antibody. Note absence of pigmentation in cambial layer cells (cl), fibroblast-like mesenchymal cells (fm), and osteocytes in the tibia (t). Bar, 85 μm.*

arrival at the fracture site. *In vitro* studies have demonstrated that macrophages are potent sources of growth factors. They secrete TGF-ß for stimulation of collagen matrix production, FGF for fibroblast proliferation without connective tissue matrix production, and PDGF for fibroblast proliferation with the production of connective tissue matrix (Kovacs, 1991).

PDGF immunoreactivity occurred at 2 days post-fracture in migrating and phagocytosing macrophages. PDGF is a potent stimulator of cell division in cells of mesenchymal origin (Bowen-Pope *et al.*, 1985). Therefore, PDGF is an ideal initiator of wound healing and in particular, fracture repair. In addition to removing dead cells, production of PDGF by macrophages may initiate cell division in and around the fracture site. Joyce *et al.* (1990a) observed that PDGF injections stimulated cell division in the periosteum. PDGF may act as a key unlocking cell division in fibroblast-like mesenchymal cells and in cells of the cambial layer of the periosteum. Once cell division has been initiated, other growth factors may maintain it.

During the response to inflammation in stage 1, a population of fibroblast-like mesenchymal cells accumulates. This accumulation continued through stage 2 of fracture repair. A dramatic increase in their number coincided with cellular activity in the cambial layer of the periosteum. This allowed these cells to form the first bridge across the fracture gap.

The cambial layer of the periosteum plays a key role in the repair process. With the decline in the number of macrophages, a potent source of growth factors was removed. The loss of PDGF was replaced by a growth factor produced by the cells of the cambial layer. Kovacs (1991) and others have noted that FGF is a potent stimulator of fibroblast proliferation. Positive staining for aFGF occurred in these cambial layer cells for as long as the cambial layer was distinct (prior to chondrification, or stage 3 of fracture repair). Canalis *et al.* (1988) reported that FGF stimulates cell replication within the periosteum and has an inhibitory effect on collagen synthesis. The immunolocalization of aFGF to the cells of the cambial layer agrees with these findings. In bridging the fracture gap, a scaffold was formed for the third stage of fracture repair, the chondrification stage.

During the third stage, the fibrous callus, consisting of fibroblast-like mesenchymal cells and cells of the cambial layer, was replaced by cartilage. A key event in chondrogenic transformation of these mesenchymal cells was increasingly close contact between neighboring cells, i.e. high density (Hall and Miyake, 1992).

By day 6 post-fracture, the fracture site was densely populated with fibroblast-like mesenchymal cells, many of which could be observed rounding up and starting to take on a chondroblastic phenotype. Cartilage appeared in small amounts on day 6 post-fracture, increasing until the fracture callus was totally chondrified by day 9 post-fracture. The amount of cartilage increased markedly at day 8 post-fracture. At this time many young chondroblasts at the edge of the cartilage stained positively for IGF-I. These young chondroblasts were actively involved in synthesis of new cartilage matrix but stained faintly with alcian blue, indicating secretion of relatively small amounts of matrix components, including chondroitin sulfate.

This growth factor was first characterized by its ability to stimulate S^{35} uptake by costal chondrocytes in tissue culture (Salmon and Daughaday, 1956), and has since been found to stimulate chondrocyte proliferation and matrix production (Bohme *et al.*, 1992). Therefore, IGF-I may assist in the production of cartilage matrix during chondrification of the fracture callus (stage 3 of fracture repair). This cartilaginous callus was only a temporary state; by day 9 post-fracture, the cartilaginous callus was undergoing replacement by new bone through endochondral ossification.

At the conclusion of the chondrogenic stage of fracture repair and prior to endochondral ossification, the cartilage matrix undergoes changes. Mineral crystals appear in the mitochondria of chondrocytes, calcium-phospholipid-phosphate is produced, and submicroscopic membrane-bound vesicles appear in the cartilage matrix (Martin and Matthews, 1970; Boskey and Posner, 1976; Anderson, 1980). In short, mature cartilage calcifies. Following calcification, blood vessels and osteogenic cells invade the cartilage mass and the process of endochondral ossification ensues (Ham and Cormack, 1979). In the present model, the process started at approximately day 9 post-fracture and continued to day 21. As bone replaced mature cartilage in the callus, chondrocytes were observed producing a calcified rim around their lacunae. These calcified lacunae appeared at the edge of the ossification front. Intense TGF-ß staining occurred in mature chondrocytes at the edge of the ossification fronts. This observation further supports the findings of Joyce *et al.* (1990b) that TGF-ß can be immunolocalized in tissues during the later stages of fracture repair.

This growth factor was originally characterized by its ability to induce a transformed phenotype (DeLarco and Todaro, 1978). Chondrocyte transformation is not a new concept; Richman and Diewert (1988) observed transformation of chondrocytes into osteoprogenitor cells and osteoblasts. Frazer *et al.* (1991) found that cultured chondrocytes release TGF-ß into the culture media. TGF-ß may be produced at this particular stage of the repair process for three reasons. First, it may signal a transformation in cell and/or matrix type in calcifying chondrocytes. Second, it may stimulate the differentiation of invading osteoprogenitor cells into osteoblast-like cells. Third, it may stimulate proliferation of these osteoprogenitor/osteoblast-like cells. The TGF-ß antibody used was produced against porcine TGF-$ß_1$ and primarily recognizes TGF-$ß_1$. However, it also reacts with other subgroups of the TGF-ß family. Consequently, positive staining for TGF-ß may indicate the presence of other TGF-ßs in addition to TGF-$ß_1$.

As summarized in Table 1, for every stage of the repair process, there was at least one cell type producing a particular growth factor. The cell types producing the growth factors are transient members of the repair process. Macrophages appear at stage 1 and produce PDGF. When the population of macrophages declines, the loss of their growth factor stimulation is replaced by another group of cells producing another growth factor, reflecting changing environmental conditions at the fracture site. Cells of the cambial layer produce aFGF to stimulate cell replication in fibroblast-like mesenchymal cells, building a sufficient population of flexible fibroblast-like cells to bridge the ends of the fracture site. When the ends of the fracture are secured, the stimulatory activity of the cambial layer cells may be replaced by still a different growth factor produced by a different cell type. Chondrogenesis, the next process to occur, requires that fibroblast-like mesenchymal cells be replaced by cartilage-producing cells. Chondroblasts produce IGF-I and may respond to it in an autocrine manner to produce cartilage matrix.

With invasion of the mature cartilage by blood vessels and osteoprogenitor cells, transformation of cartilage to bone began. This transformation took place at the edge of the cartilage mass along the ossification front. TGF-ß signalled this transformation within the cartilage mass possibly by initiating calcification of the

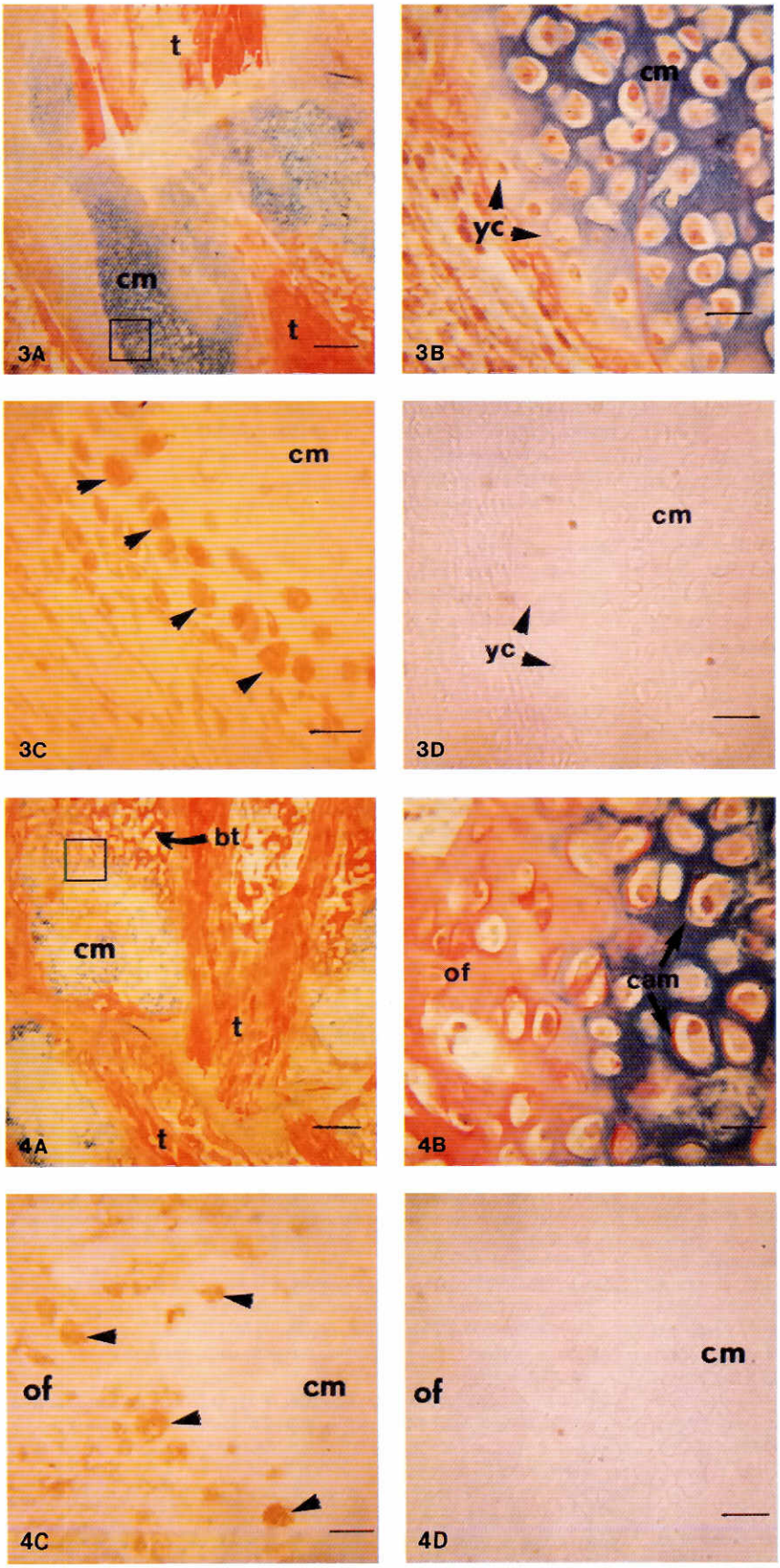

Fig. 3. Immunohistochemical detection of IGF-I during stage 3 of fracture repair. (A) *A low-power photomicrograph of a fracture site at day-8 post-fracture stained by the HBQ method. Note area of cartilage mass (cm) and tibia (t). Bar, 192 μm.* **(B)** *A high-power photomicrograph of area in inset from (3A). The area highlighted is the edge of the cartilage mass (cm). Note weakly staining young chondroblasts (yc) at the periphery of the cartilage tissue. Bar, 21 μm.* **(C)** *Serial adjacent section to one in (3B) with polyclonal antibodies to IGF-I applied. The chondroblasts at the periphery of the cartilage stain positive for IGF-I (arrow heads), while chondrocytes within the cartilage mass (cm) do not. Bar, 21 μm.* **(D)** *Serial adjacent section to one in (3C) omitting primary IGF-I antibody. Note absence of pigmentation in cartilage mass (cm) or young chondroblasts (yc). Bar, 85 μm.*

Fig. 4. Immunohistochemical detection of TGF-ß during stage 4 of fracture repair. (A) *A low-power photomicrograph of a fracture site at 14 days post-fracture stained by the HBQ method. Present in the photograph are the tibia (t) and new bone trabeculae (bt) seen infiltrating and replacing the cartilage mass (cm). Bar, 192 μm.* **(B)** *A high-power photomicrograph corresponding to the area in inset from (4A). The area outlined is the periphery of the remaining cartilage. Note the ossification front (of) moving from left to right. Also note that chondrocytes at the edge of the ossification front are producing a red rim (cam) inside their lacunae signifying the production of a calcified matrix. Bar, 21 μm.* **(C)** *Serial adjacent section to one in (4B) with polyclonal antibodies to TGF-ß applied to it. Note the positively staining calcified chondrocytes at the edge of the ossification front (arrow heads). Note absence of staining on chondrocytes toward the center of the cartilage mass (cm). Bar, 21 μm.* **(D)** *Serial adjacent section to one in (4C) omitting primary TGF-ß antibody. Note absence of pigmentation in cells of the cartilage mass (cm) or ossification front (of). Bar, 85 μm.*

TABLE 1

IMMUNOHISTOCHEMICAL LOCALIZATION OF SELECTED GROWTH FACTORS IN VARIOUS CELL TYPES AT FOUR STAGES[a] OF FRACTURE REPAIR

Growth Factor	Macrophages	Cell Type: Cambial Layer	Fibroblast mesenchymal	Chondroblast	Osteoblast
		Stage 1			
PDGF	+	-	-	not present[b]	not present
		Stage 2			
aFGF	-	+	-	-	not present
		Stage 3			
IGF-I	not present	-	-	+	-
		Stage 4			
TGF-ß	not present	-	-	-	+

[a]For histological characteristics of the four stages see text.
[b]Not present means that the cell type was not present at that stage.

cartilage matrix. Chondrocytes at the edges of the cartilage mass produced TGF-ß until the ossification front moved past them and they became enclosed in new bone. TGF-ß production then ceased in these former chondrocytes and was initiated by chondrocytes where the ossification front was now located. It should be noted, however, that only four growth factors were analyzed in this fracture model. Consequently, the experimental results obtained offer only a partial explanation of the role of growth-stimulating molecules in bone physiology. Other growth factors and bone inductive proteins such as bFGF, TGF-ßs$_{2\text{-}3}$ and BMP are also active participants in bone physiology. Further studies are needed in order to decipher this complex system.

Materials and Methods

Animals and preparation of the specimens for immunohistochemistry

Thirty-three male CD-1 mice were fractured according to the procedure outlined in Bourque *et al.* (1992). Three mice were harvested per day from day-1 post-fracture to day-10 and three more mice from day-14. Animals were anesthetized and euthanized. Fractured tibiae were removed, fixed in periodate-lysine-paraformaldehyde, decalcified in ethylenediaminetetraacetic acid-glycerol, dehydrated through isopropanol and infiltrated with low melting point paraffin according to the procedure outlined in Bourque *et al.* (1993). After embedding, tissues were sectioned on a microtome at 5 µm and analyzed immunohistochemically for the presence of growth factors.

Antibodies

Polyclonal neutralizing IgG antibodies to human PDGF and porcine TGF-ß$_1$ raised in goats and rabbits respectively and purified by Protein G affinity chromatography were purchased from R&D systems (Minneapolis, MN, USA). Polyclonal IgG antibodies to aFGF fragment (leu60-leu98) raised in rabbits and purified by Affi-gel blue column chromatography were ordered from Genzyme (Cambridge, MA, USA). Polyclonal anti-human IGF-I antibodies raised in rabbit, and purified by precipitation with ammonium sulfate followed by ion-exchange chromatography, were from KabiGen (Stockholm, Sweden). Biotinylated rabbit anti-goat and goat anti-rabbit antibodies and normal rabbit and goat serums were purchased from Vector Laboratories (Burlingame, CA, USA). Hydrogen peroxide (H_2O_2) and Diaminobenzadine (DAB) were received from Sigma (St. Louis, MO, USA).

Immunohistochemical procedure

Sections were deparaffinized and stained by the avidin-biotin-peroxidase complex (ABC) immunoperoxidase technique according to Hsu *et al.* (1981) and modified by Bourque *et al.* (1993). Sections were immersed in 3% hydrogen peroxide for 5 min followed by washing in running water for 5 min and flooding with PBS for 5 min. Then they were incubated with 100% (v/v) normal goat serum (aFGF, TGF-ß, IGF-I) or normal rabbit serum for (PDGF) for 30 min at room temperature. Sections were drained well and incubated with primary antibody diluted with PBS (containing 0.5% bovine serum albumin) in the ratio of 1:500 (aFGF and PDGF) or 1:100 (TGF-ß and IGF-I) overnight at 4°C. After washing with PBS, they were incubated with secondary antibody (biotinylated goat anti-rabbit IgG for aFGF, TGF-ß, IGF-I; biotinylated rabbit anti-goat for PDGF) diluted with PBS in the ratio of 1:200 for 30 min at room temperature. Washing with PBS was followed by incubation with avidin-biotin-peroxidase complex from ABC Elite Kit PK-6100 (Vector; Burlingame, CA, USA) for 30 min at room temperature. After washing with PBS, brown pigmentation was produced by 2 min treatment with 3,3'-diaminobenzidine (DAB) (Sigma, St. Louis, MO, USA) (1 DAB tablet dissolved in 20 ml 0.05 M Tris-HCl buffer, pH 7.6, containing 0.005% hydrogen peroxide). As a negative control, PBS was substituted for the primary antibodies.

Serially adjacent sections to those studied immunohistochemically were stained with the HBQ method (Hall, 1986). The HBQ staining procedure uses the histochemical stains, direct red, alcian blue, Mayer's hematoxylin, and celestian blue B, to provide excellent contrast between bone (stains red), cartilage (stains blue) and connective tissue.

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