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The Role of Chondrocytes in Intramembranous and Endochondral Ossification During Distraction Osteogenesis in the Rabbit

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Abstract. We have used a rabbit leg-lengthening model for detailed studies of the histology of distraction osteogenesis. Some unusual features of the endochondral ossification that occurs during the rapid transition of cartilage to bone in the regenerate were observed. Histological staining techniques together with immunohistochemistry and nonradioactive *in situ* mRNA hybridization for cartilage and bone-related molecules have been used to document the presence of an overlapping cartilage-bone phenotype in cells of the cartilage-bone transitional region. In those particular areas, some chondrocytes appeared to be directly transformed into newly formed bone trabeculae which are surrounded by bone matrix. Acid phosphatases were found within the cartilage matrix in some of the cartilage/bone transitional regions and type I collagen mRNA and type II collagen protein were found together in some of the marginal hypertrophic chondrocytes. This study indicates an unusual role of chondrocytes in the process of ossification at a distraction rate of 1.3 mm/day in the rabbit. Further direct evidence is required to prove the hypothesis that the hypertrophic chondrocytes may transdifferentiate into bone cells in this model.

Key words: Hypertrophic chondrocyte — Transdifferentiation — Distraction osteogenesis — Rabbit model — Collagen — *In situ* hybridization — Immunohistochemistry.

Induction of osteogenesis by means of an osteotomy, followed by fixation with an external fixator and subsequent controlled distraction of the callus, is a developing technique with widespread clinical application in the treatment of bone defects, limb deformities, and fracture nonunions [1, 2]. Previous reports on distraction osteogenesis at low rates of distraction (0.5–1.0 mm/day) in the human [3, 4], dog [5–7], and sheep [8, 9] show that the bone forms by intramembraneous ossification and no endochondral ossification is observed. However, in the rabbit model of leg lengthening, direct cartilage formation from the central fibrous tissue of the regenerate is observed [10, 11].

We have used a rabbit leg-lengthening model for detailed studies of the histology of the tissues formed by distraction osteogenesis. Using specific matrix stains (alcian blue/sirius red) [12], enzyme detection (acid phosphatase), immunohistochemical detection of type II collagen protein, and the detection of the mRNA expression of type I collagen gene by *in situ* hybridization technique, we have observed some unusual morphological features of the endochondral ossification that occurs during the transition of cartilage to bone in distraction osteogenesis.

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Materials and Methods

Animal Model of Leg Lengthening

Six New Zealand white rabbits (aged 24 weeks, body weight 3.0–3.5 kg) were anesthetized by intramuscular injection of Hypnorm (0.2 ml/kg) (Janssen Animal Heath, High Wycombe, England), intravenous injection of hypnovel (2 mg/kg), Roche, Welwyn Garden City, England), and locally infiltrated with 0.25% Marcain (Astra Pharmaceutical Ltd, England). A 4-cm incision was made over the medial aspect of the left tibia, and an Orthofix M-100 lengthener (Orthofix, Italy) was fixed with four stainless-steel screws inserted in the tibia. The tibia was osteotomized at the tibiofibular junction between the two inner screws using a hand saw under saline irrigation. Seven days after surgery, the tibia was lengthened at a rate of 1.33 mm/day by turning the lengthener by one turn (0.67 mm linear displacement) every 12 hours for 15 days. Weekly radiography confirmed the lengthening rate.

Sample Preparation and Histological Examination

The rabbits were killed by anesthetic overdose at the end of the experiment and the central portion of the distraction gap, the regenerate, was excised and fixed in 4% (w/v) buffered paraformaldehyde for 24 hours before decalcification in neutral EDTA (14.5%, pH 7.2) at room temperature for 14–30 days. The regenerate was embedded in paraffin wax and 5 μm sections were cut by microtome and placed on poly-L-lysine-(Sigma, Poole, UK) coated slides for histology and immunohistochemistry or on 3-aminopropyltriethoxy silane-(Sigma) coated slides for *in situ* mRNA hybridization. The sections were dewaxed with xylene and rehydrated in four sequential ethanol baths from 99% to 50% ethanol, each for 5 minutes. For histological examination, the sections were stained with hematoxylin and eosin (H&E staining). Weigerts hematoxylin/alcian blue/sirius red stain [12] was used to distinguish bone matrix (red) from cartilage matrix (blue).

Tartrate-Resistant Acid Phosphatase Staining

For identifying bone-resorbing cells, tartrate-resistant acid phosphatase (TRAP) staining was performed. In brief, paraffin sections were dewaxed and rehydrated through ethanol, washed in Tris-HCL buffer (pH 9.0), and incubated with acetate buffer (0.5% w/w sodium acetate, 0.83% v/v acetic acid, pH 5.0) for 1 hour. The

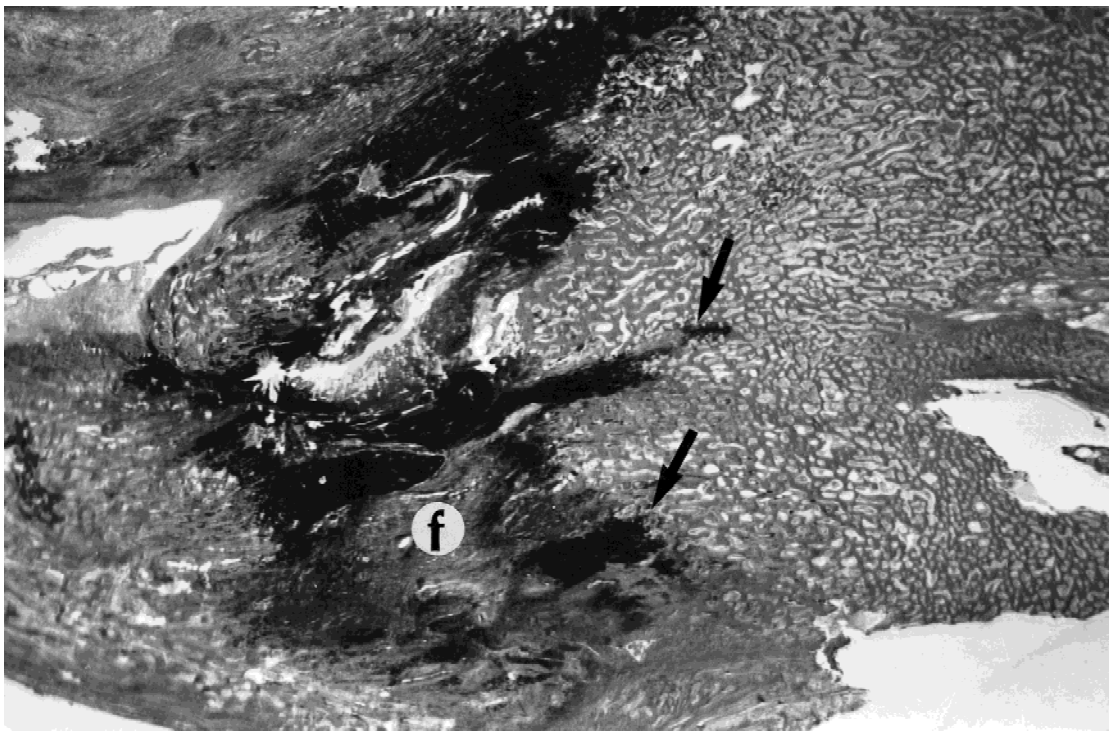

Fig. 1. Representative of morphological appearance of the distraction regenerate. The regenerate contains a central fibrous zone (f) and moderate amounts of cartilage (arrows). Alcian blue and sirius red staining, ×8.

substrate mixture consisted of 15 mg Naphthol AS B1 phosphate (Sigma), 75 mg sodium tartrate, 1 mg pararosaniline, and 0.8 mg sodium nitrite in 26 ml acetate buffer (pH 5.0). The substrate mixture was filtered before use and sections were incubated with the mixture at 37°C for 1 hour. Sections were rinsed in running water for 5 minutes and counterstained by Gill's hematoxyline (Sigma) for 15 seconds, and mounted in aquamount (BDH, Poole, England).

Immunohistochemistry of Type II Collagen

Specific goat antibodies to rabbit type II collagen [13] were used for immunohistochemistry. In brief, the sections were rehydrated in tris-buffered saline, pH 7.3, for 15 minutes at room temperature and incubated in 2% hyaluronidase (ovine testicular, Sigma) in phosphate-buffered saline (PBS) for 1 hour at 37°C. They were then washed in PBS and incubated with heat-inactivated normal rabbit serum for 15 minutes at room temperature. The specific goat anti-rabbit type II collagen antibodies was applied at a dilution of 1:40 in PBS and incubated overnight at 4°C. After washing, the sections were incubated with rabbit anti-goat IgG conjugated with alkaline phosphatase for 1 hour at room temperature. Sections were then treated with visualizing substrates, X-phosphate, and NBT mixture according to the manufacturer's instruction (Boehringer-Mannheim, East Sussex, UK) for 30 minutes. The sections were then washed in PBS and mounted in glycerine jelly. Control sections were taken by the same procedure but without the primary antibodies.

In Situ Hybridization of Type I Collagen mRNA

The messenger RNA (mRNA) for type I collagen was detected by nucleic acid hybridization. Riboprobes for type I collagen mRNA were made from a cDNA probe, pHCAL1U (gift from E. Vuorio, University of Turku, Turku, Finland). To generate the antisense probe, the plasmid was linerized with HindIII and incubated with T7 polymerase; for the sense probe, it was linerized with EcoRI and incubated with SP6 polymerase. The riboprobes were labeled with digoxygenin according to the protocols provided with the labeling kit (Boehringer Mannheim, GmbH, Germany).

The protocol for *in situ* hybridization was as described by Sandberg and Vuorio [20] with some modifications. Briefly, the rehydrated sections were pretreated with 0.2 N hydrochloric acid for 20 minutes at room temperature before incubation with proteinase K at 20 μg/ml in 0.1 M Tris buffer (pH 8.0) and 50 m M EDTA for 10 minutes at 37°C. This was followed by fixation in 4% paraformaldehyde in PBS for 20 minutes on ice and washing with 7.5 mg/ml of glycine in PBS for 20 minutes. The tissues were then acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 minutes. The sections were dehydrated through graded alcohol and dried sections were then hybridized with a solution consisting of 50% formamide, 10 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, 0.02% polyvinyl pyrrolidone ficoll, 0.02% bovine serum albumin, 0.5% sodium dodecyl sulfate, and digoxygenin-labeled probe; 40 μl of the mixture was applied to each slide and covered with coverslip. After overnight incubation at 55°C, the coverslips were removed with 2 × SSC (standard saline citrate) and the sections were washed twice with 0.5 × SSC (55°C, 5 minutes each) and 0.1 × SSC at 55°C for 5 minutes. Anti-digoxygenin antibody was incubated with the sections at room temperature for 1 hour prior to development of the color by alkaline phosphatase substrates (Boehringer Mannheim, East Sussex, UK) for 2–4 hours in the dark. No counterstaining was used and the sections were mounted in glycerine jelly.

Results

The regenerate contains a central fibrous zone, mainly fibroblastic cells; the new bone developed from the central fibrous zone and extended to both osteotomized ends. The newly formed bone was highly oriented toward the distrac-

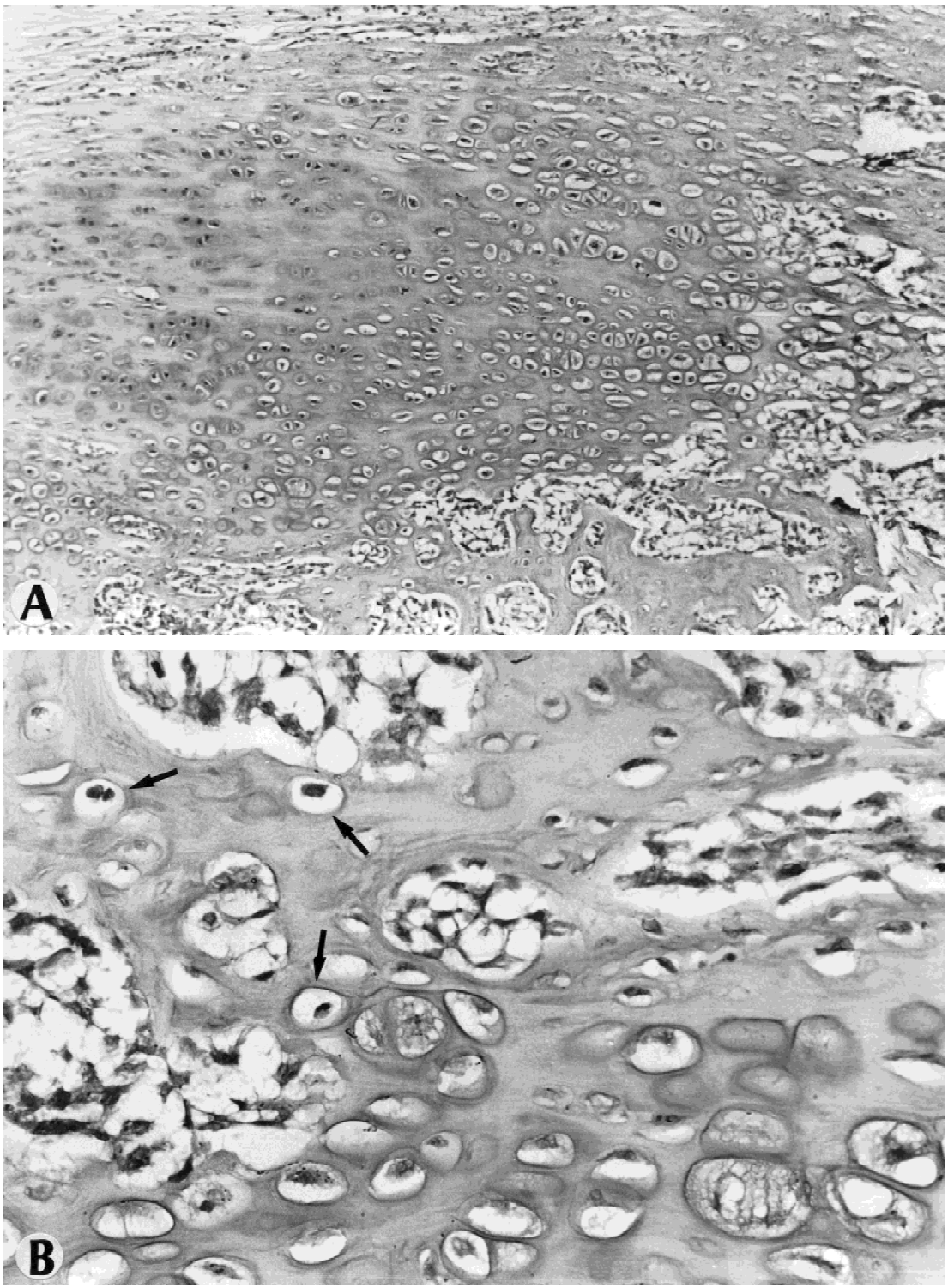

Fig. 2. (A) H & E staining showing the cartilage developing from the central fibrous tissue. Note typical endochondral ossification at the cartilage/bone transitional region. ×100. **(B)** High power view of the cartilage/bone transitional regions. Some chondrocytes remain intact in their lacunae within the new woven bone trabeculae (arrows). H & E staining, ×400.

tion forces (Fig. 1). Moderate amounts of cartilage within the regenerate were found and the cartilage arose directly from the central fibrous tissue in some regions (Fig. 2A), extending to new bone trabeculae without distinct boundaries (Fig. 2B). Cartilage remnants were often seen in the new bone trabeculae, where chondrocytes were buried into bone matrix, as demonstrated by alcian blue and sirius red staining (Fig. 3A, B). TRAP-positive cells were seen in most areas of the cartilage-bone junction and the TRAP-positive staining was also observed in the cartilage matrix in areas of cartilage-bone transitional interface (Fig. 3C).

Small areas of cartilage were also found adjacent to the

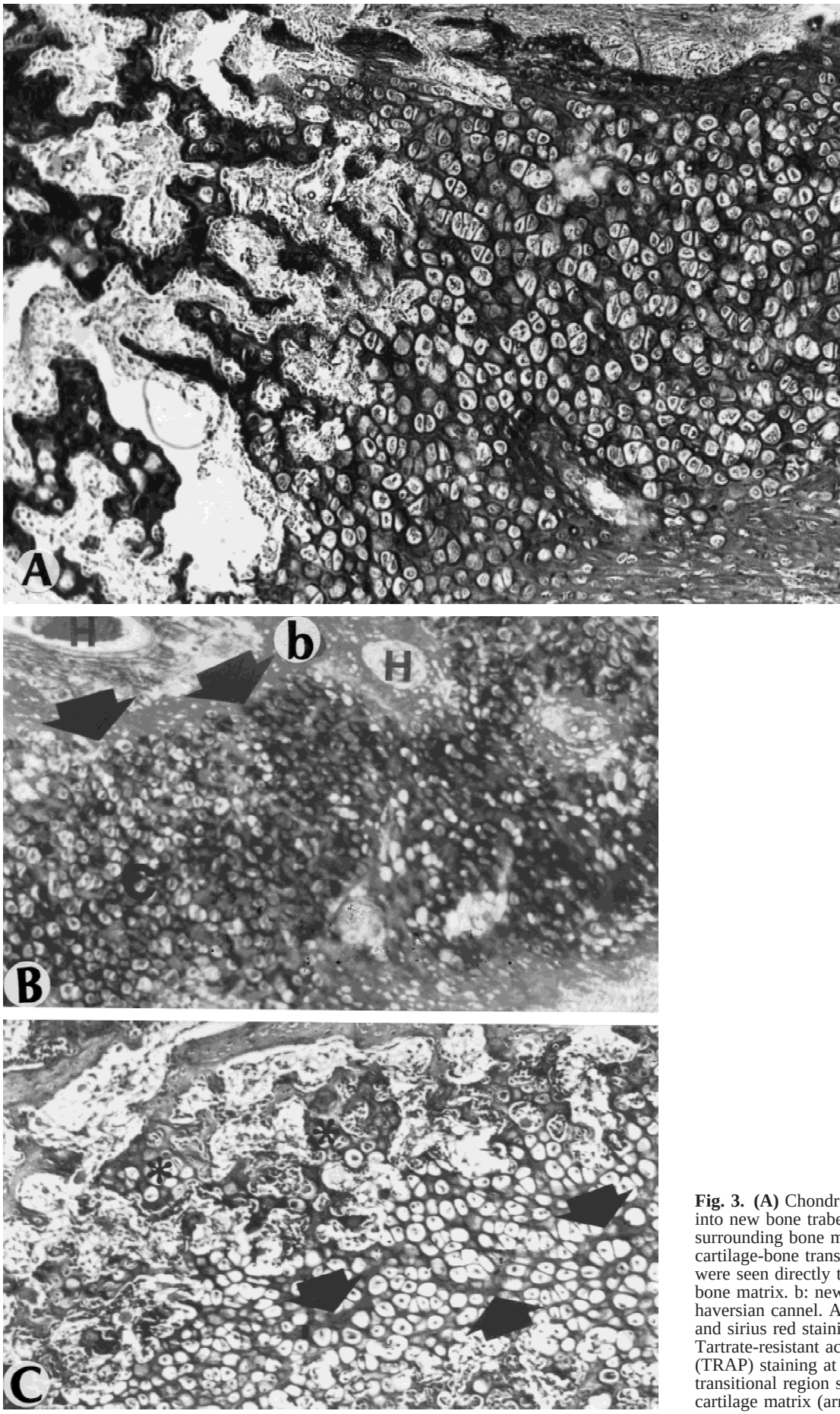

Fig. 3. (A) Chondrocytes transforming into new bone trabeculae with surrounding bone matrix. **(B)** In areas of cartilage-bone transition, chondrocytes were seen directly transforming into new bone matrix. b: new bone; c: cartilage; H: haversian cannel. A and B, alcian blue and sirius red staining, ×100. **(C)** Tartrate-resistant acid phosphatase (TRAP) staining at the cartilage-bone transitional region showing staining in the cartilage matrix (arrows), ×100.

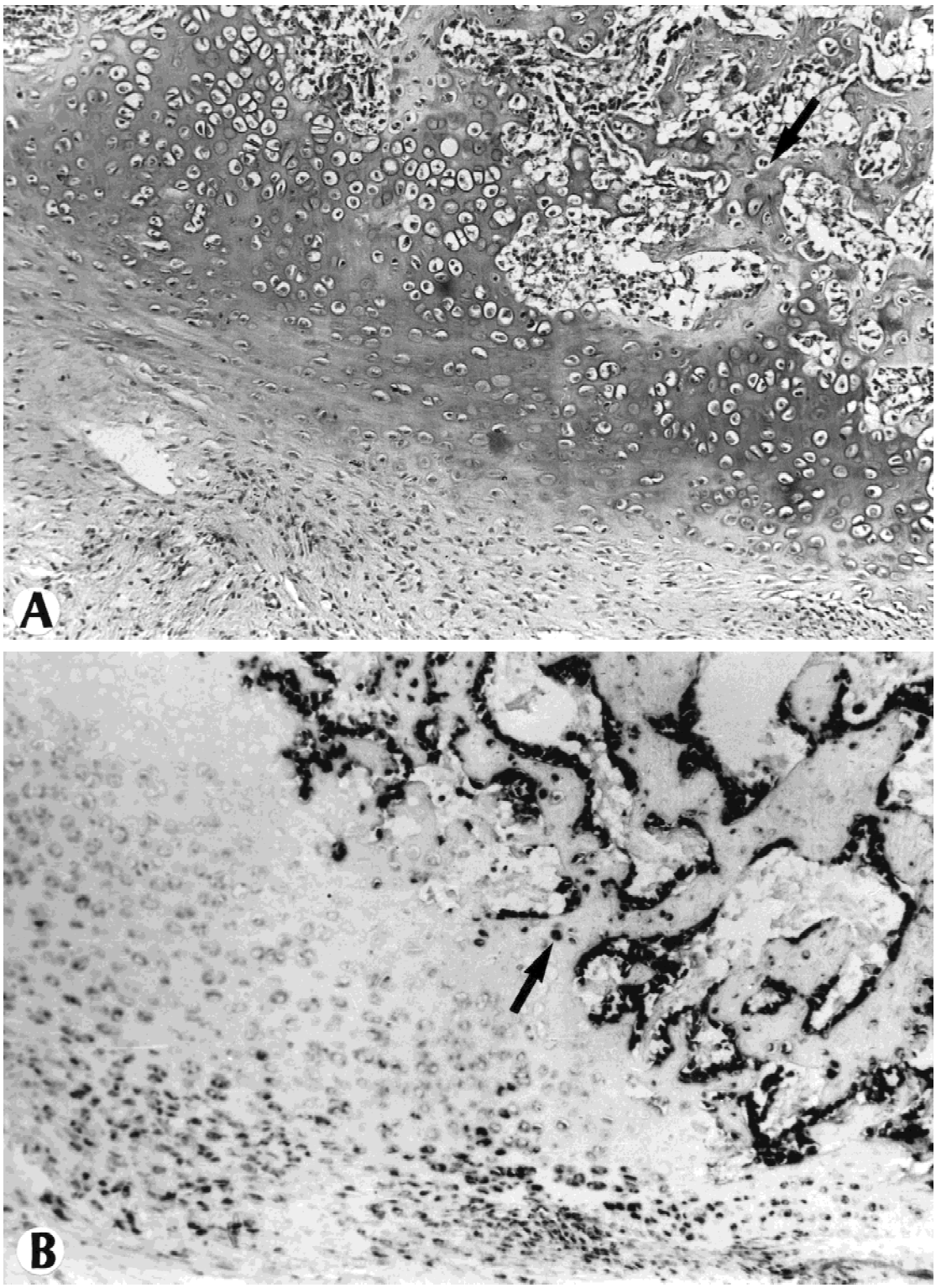

Fig. 4. (A) H&E staining shows the region where cartilage was developing from fibrous tissue. Note the cartilage remnants in the new bone trabeculae (arrow). **(B)** *In situ* hybridization showing the type I collagen mRNA present in the newly forming chondrocytes at the subperiosteal region, and diminishes as the chondrocytes differentiate further and became hypertrophic. Type I collagen mRNA is also detected in the cartilage remnants within the new trabeculae (arrow). A and B, ×100.

periosteum, where cartilage was developing from the periosteal cells to form an intermediate step in new bone formation at the site. In some bone-cartilage transition regions, cells with chondrocyte morphology appeared to transform into new bone trabeculae (Fig. 4A). *In situ* hybridization with type I collagen mRNA antisense probe showed that the cells with the strongest expression of type I collagen mRNA were localized on the new bone surfaces; these cells were

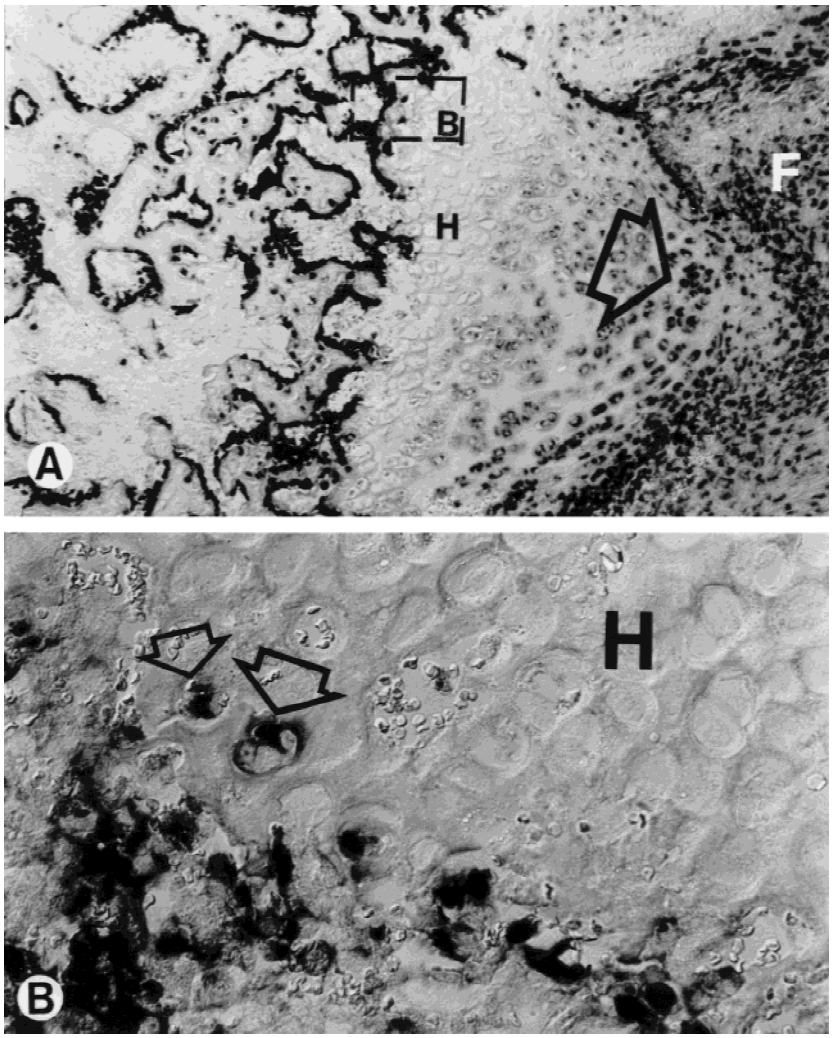

Fig. 5. (A) *In situ* hybridization with type I collagen antisense mRNA probe. The type I collagen mRNA is strongly expressed by the chondrocytes at the fibrous-cartilage transitional region (arrow), the expression diminishes as the chondrocytes differentiated and became hypertrophic, and type I collagen mRNA is detected again in a few hypertrophic chondrocytes at the cartilage-bone transitional region **(B).** F: fibrous tissue; H: hypertrophic chondrocytes; ×100. **(B)** Close-up of the boxed area in **(A)** Type I collagen mRNA is present in a few hypertrophic chondrocyte lacunae (arrows), ×400.

flattened and were cuboidal multilayered preosteoblasts or osteoblasts. A few newly formed osteocytes in the core of bone trabeculae expressed type I mRNA also, but the signal was weaker and diminished as the woven bone completed calcification (Figs. 4B and Fig. 5A). In the region of fibrous-cartilage transition, the cartilage developed from the fibrous tissue which expressed type I collagen mRNA (Fig. 4A and Fig. 5A). The type I collagen mRNA still remained in the newly forming chondrocytes in the transitional margin between the fibrous tissue and the cartilage. With further differentiation of the chondrocytes, type I collagen mRNA expression was greatly reduced and the chondrocytes became hypertrophic (Figs. 4B and Fig. 5A). At the transitional region between cartilage and bone, type I collagen mRNA was detected in a small number of cells within the hypertrophic chondrocyte region (Fig. 5B). In addition to *in situ* hybridization with type I collagen mRNA probe, immunohistochemistry using specific antibody to type II collagen was applied to adjacent sections; the presence of type II collagen protein in these cells confirmed that the marginal cells between cartilage and bone were indeed chondrocytes and that they were expressing type I collagen mRNA (Fig. 6A–C).

Controls for type II collagen immunostaining were performed without applying primary antibody. Hybridization using the sense probe of collagen type I was used as control for the *in situ* hybridization procedure. The controls were all negative, with only minimum background staining observed (not shown).

Discussion

Previous studies on distraction osteogenesis in human, dog, and sheep species were carried out at rates of distraction between 0.5 and 1.0 mm/day. Bone formation by intramembraneous ossification was observed to be the unique characteristic of distraction osteogenesis in these species. However, in rabbits, cartilage was observed during tibial or femoral lengthenings by using Orthofix unilateral lengtheners at rates of 0.5–1.0 mm/day [10, 11]. This difference may be due to species differences in response to distraction or it may be a result of instability of the fixed osteotomy.

In the present study, we have used a rabbit model of leg lengthening similar to that described by Kojimoto et al. [10] and found that moderate amounts of cartilage are present in the distraction regenerate, with a mixture of endochondral and intramembraneous ossification occurring. Resultant instability of the osteotomy at this distraction rate (1.3 mm/day) cannot be ruled out and the cartilage formation may suggest interference with the blood supply to the regenerating tissues at the relatively high rate.

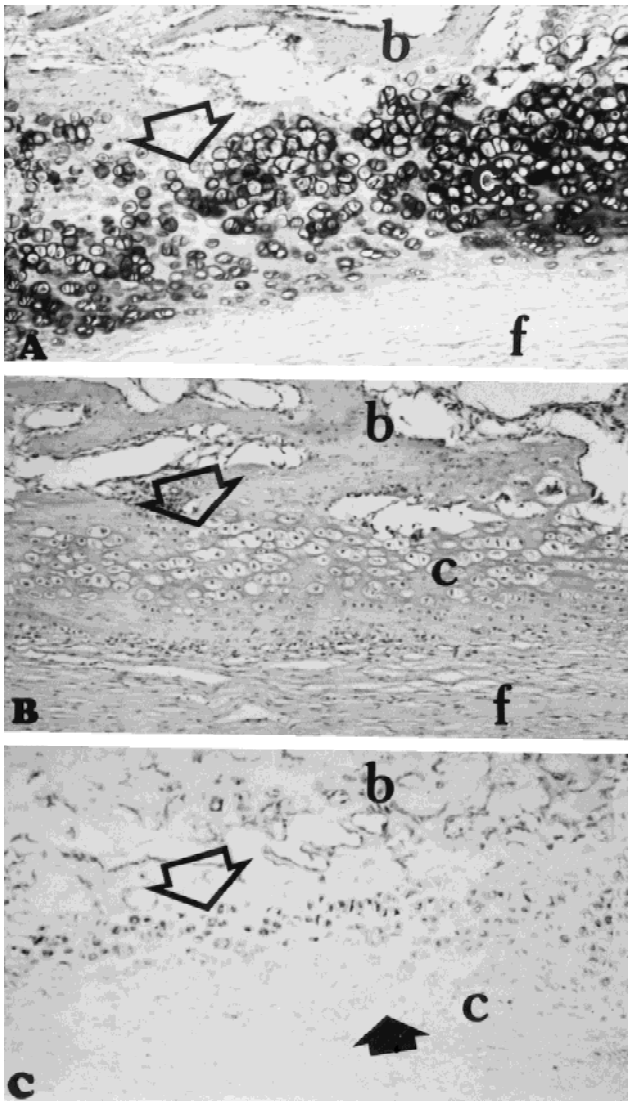

Fig. 6. (A) H&E staining shows that the cartilage (arrow) is present between fibrous tissue and new bone. **(B)** Immunostaining using anti-rabbit type II collagen antibody. The chondrocytes at the transitional region are synthesizing type II collagen proteins (arrow). **(C)** *In situ* hybridization using type I collagen antisense probe. Type I collagen mRNAs are detected in the chondrocytes at the upper layer of the cartilage-bone transitional region (empty arrow), but not at the middle layer of the cartilage (solid arrow). b: bone; c: cartilage; f: fibrous tissue. A, B, and C, ×100.

Two histological staining techniques, immunohistochemistry and nonradioactive *in situ* hybridization, have been used to document the presence of an overlapping cartilage/bone phenotype in cells of the cartilage/bone transition region. In these region, we observed that some chondrocytes appeared to be directly transformed into newly formed bone trabeculae surrounding by bone matrix. This was clearly shown by alcian blue/sirius red staining and this is termed "chondroid bone" [14]. When the undifferentiated osteogenic progenitor cells in the newly formed skeletal tissue are stimulated to differentiate, some do not go down a simple pathway to an osteoblast or chondrocyte. Because of their environment and systemic and local factors, some cells may appear to become "ambivalent" and may become hybrid cells producing chondroid bone or fibrocartilage, which may produce matrices with both types I and II collagens (D. Ashhurst, personal communication).

An interesting observation was the presence of acid phosphatase within the cartilage matrix in the cartilage/bone transition region. Similar observations have been reported by Scammell and Roach [15] in a study of fracture healing in the rabbit, although the contribution of enzyme diffusion during reaction procedure has not been completely investigated. They found acid phosphatase activity within intact chondrocytic lacunae and they defined this as "lacunar" bone. They suggest a role for chondrocytes in fracture repair, with endochondral ossification, including direct bone formation by former chondrocytes. Robles-Marin et al. [16] reported an increase of acid phosphatase activity in the chondrocytes next to the vascular invasion during endochondrial ossification. They proposed that vascular invasion will occur only after the secretion of acid phosphatases from the chondrocyte into the cartilage matrix. Disintegration of the cartilage matrix in the distraction regenerate is proposed to occur by oxidation following vascularization.

At the cartilage-bone transitional regions, type I collagen mRNA and type II collagen protein were found together in some of the marginal hypertrophic chondrocytes. This could be a result of direct transformation of hypertrophic chondrocytes into osteoblasts or a result of invasion of the chondrocyte lacunae by osteoblast progenitor cells, but it is impossible to distinguish between these two hypotheses from the current study. Previous studies of chondrocyte differentiation have indicated that hypertrophic chondrocytes *in vitro* may express characteristic proteins for the osteoblast phenotype [4]. Expression of these proteins by hypertrophic chondrocytes has also been observed *in vivo* in selected cartilage regions. *In situ* hybridization and/or immunohistochemistry shows that human, rat, and chicken hypertrophic chondrocytes may be capable of synthesizing of type I collagen [8, 17, 18, 19, 20, 21], osteocalcin [18, 21], osteopontin [22], and osteonectin [23, 24]. In addition, Galotto et al. [25] have shown that preosteoblasts and osteocytes share the same membrane protein as hypertrophic chondrocytes. Nevertheless, none of these published observations conclusively prove that hypertrophic chondrocytes give rise to osteoblasts directly.

An alternative explanation for the presence of type I collagen mRNA in the chondrocytic lacunae is that these lacunae may be invaded by other cells outside the plane of section. They are likely to be osteogenic progenitor cells brought into the chondrocyte lacunae by the invading blood vessels.

In conclusion, morphological observations, together with the pattern of acid phosphatase activity, expression of type I collagen mRNA, and localization of type II collagen protein, indicate a process of bone ossification via cartilage progenitors at higher rates of distraction in the rabbit. The terminal fate of hypertrophic chondrocytes is still controversial and it has been suggested that it may be different at different microanatomical sites [26]. Previous reports regarding chondrocyte and osteoblast turnover, together with the present findings, suggest that osteoblasts and chondroblasts originate from a common pool of mesenchymal cells. These differentiated cells could share some plasticity of phenotype. However, in this report, as in all other studies published to date, more direct evidence is required before any claims can be made that the hypertrophic chondrocytes may transform into bone-forming cells.

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