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TISSUES FORMED DURING DISTRACTION OSTEOGENESIS IN THE RABBIT ARE DETERMINED BY THE DISTRACTION RATE: LOCALIZATION OF THE CELLS THAT EXPRESS THE mRNAs AND THE DISTRIBUTION OF TYPES I AND II COLLAGENS

G. LI[1,2]*, A. S. VIRDI[2]**, DOREEN E. ASHHURST[3], A. H. R. W. SIMPSON[1] and J. T. TRIFFITT[2]

[1]*Nuffield Department of Orthopaedic Surgery &* [2]*Bone Research Laboratory, Nuffield Orthopaedic Centre, Headington, Oxford, OX3 7LD, U.K.* [3]*Department of Anatomy, St. George's Hospital Medical School, London, SW17 0RE, U.K.*

*Present addresses *Orthopaedic Research Unit, Musgrave Park Hospital, Belfast, BT9 7JB, U.K. **Department of Anatomy, Rush-Presbyterian-St. Luke's Medical Center, Rush University, Chicago, IL60612-3832, U.S.A.*

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An experimental model of leg lengthening was used to study the morphology of, the collagenous proteins present, and the collagen genes expressed in the regenerating tissue following 20% lengthening at four different distraction rates. At a distraction rate of 0.3 mm/day (8 weeks distraction), the regenerate consists of intramembranous bone and localized areas of fibrocartilage. At rates of 0.7 (4 weeks) and 1.3 mm/day (2 weeks), the bone that grows from the cut ends of the cortical bone is separated by fibrous tissue and cartilage is present. At 2.7 mm/day (1 week), only fibrous tissue and sparse bone are present. Type I collagen is present in the matrices around the cells expressing its mRNA and similarly, type II collagen is located around the chondrocytes. Type I collagen mRNA is expressed predominantly by the fibroblasts in the fibrous tissue, the bone surface cells and to a reduced extent by the osteocytes. Type II collagen mRNA is expressed by chondrocytes. The results suggest that osteoblasts and chondrocytes within the regenerate originate from the same pool of progenitor cells, and the differentiation of these cells and the expression of types I and II collagen genes are altered by different rates of distraction. These observations suggest that the optimal rate of distraction in the model is 0.7 mm/day.

KEYWORDS: distraction osteogenesis; collagen; immunohistochemistry; *in situ* hybridization.

INTRODUCTION

Induction of osteogenesis by means of an osteotomy followed by controlled distraction of the callus is a useful technique with widespread clinical application in the treatment of bone defects, limb deformities and fracture non-unions (Kawamura *et al.*, 1968; Coleman and Stevens, 1978; Ilizarov, 1990). The quality of osteogenesis in limb lengthening is largely determined by the surgical procedures used. The preservation of the soft tissues and periosteum around the osteotomy site, although of major importance, does not alone guarantee the success of distraction osteogenesis. The amount, rhythm or frequency, and rate of lengthening are also of great importance. Ilizarov (1989b) investigated the influence of both rate and frequency of distraction on osteogenesis using a dog model and he found that a distraction rate of 1.0 mm/day in four steps or auto-distraction at 0.017 mm every 24 min led to the best bone formation. Our previous studies suggest that distraction rates of 0.3–0.7 mm/day are beneficial for both muscle regeneration and angiogenesis in the

*To whom correspondence should be addressed. Dr D. E. Ashhurst, Department of Anatomy, St George's Hospital Medical School, Tooting, London SW17 0RE, U.K. Tel: 0181 725 5224; Fax: 0181 725 3326. E-mail: d.ashhurst@sghms.ac.uk

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developing bone (Simpson *et al.*, 1995; Li *et al.*, 1999). Clinically, distraction at a rate of 1.0 mm/day in 2–4 steps is considered most favourable to osteogenesis and is commonly applied.

Despite adequate bone regeneration, the Ilizarov technique still produces an overall complication rate of ~30% (Garcia-Cimbrelo *et al.*, 1992). In the past, leg lengthening was a procedure of last resort because of its high complication rate, significant morbidity, and long period of disability even without complications (Moseley, 1989). An increased understanding of the biology of distraction osteogenesis, as well as technical advances, has now made lengthening more feasible.

The present study was designed to determine the maximal rate for beneficial distraction osteogenesis in a rabbit model and to investigate the differences in the morphology of the regenerating tissues at different rates of distraction, particularly those higher rates that have not been used previously. To confirm the distribution of bone and cartilage in the regenerates and to compare the effect of different rates on cellular differentiation leading to the formation of specific tissues, the distributions of types I and II collagens and the cells that express their mRNAs were determined.

MATERIALS AND METHODS

Animal model of leg lengthening

New Zealand white rabbits (n=16, age 24 weeks, body weight 3.0–3.5 kg) were anaesthetized and open transverse osteotomies performed in the left mid-tibial diaphysis, as previously described (Kojimoto *et al.*, 1988; Li *et al.*, 1996). The osteotomies were stabilized with unilateral external fixators (Orthofix M100, Italy). After a 7-day latency period, the osteotomies were distracted twice daily at rates of 0.3 mm/day (half turn/day), 0.7 mm/day (1 turn/day), 1.3 mm/day (2 turns/day) and 2.7 mm/day (4 turns/day), until lengthening equal to 20% of the total tibial length was achieved, as determined radiographically. The rabbits were killed by anaesthetic overdose the day after distraction ceased. Each group contained four rabbits.

Sample preparation and histological examination

The central portion of the tibia including the distraction gap was excised and halved longitudinally in the median plane. It was fixed in 4% (w/v) buffered paraformaldehyde for 24 h before decalcification in neutral EDTA (14.5%, pH 7.2) for 14–30 days. The end-point of decalcification was determined radiographically. The tissue was dehydrated in graded ethanols, cleared in xylene and embedded in paraffin wax. Sections (5 μm) of the whole distraction gap were cut and mounted on poly-L-lysine (Sigma, Poole, Dorset, U.K.) coated slides for histology and immunohistochemistry, or 3-aminopropyltriethoxysilane (APES) (Sigma, Poole, Dorset, U.K.) coated slides for *in situ* hybridization. For histological examination, the sections were stained with haematoxylin and eosin.

Immunohistochemistry for types I and II collagens

Affinity purified specific goat antibodies to rabbit types I and II collagens (Page *et al.*, 1986) were used for immunohistochemistry. The sections were rehydrated and washed with Tris buffered saline (TBS), pH 7.3, for 15 min at room temperature and incubated in 2% hyaluronidase (ovine testicular, Sigma, Poole, U.K.) in TBS for 1 h at 37°C. They were washed in TBS and incubated with undiluted heat-inactivated normal rabbit serum for 15 min at room temperature. The serum was drained from the sections and replaced by an anti-rabbit collagen antibody at appropriate dilution and incubated overnight at 4°C. After washing with three changes of TBS, the sections were incubated with rabbit anti-goat IgG, conjugated with either alkaline phosphatase or peroxidase, for 1 h at room temperature. The bound antibodies conjugated with alkaline phosphatase were located using the following substrate; 5 mg naphthol AS-BI phosphate was dissolved in 1 drop dimethyl formamide and added to 5 mg Fast Red TR in 10 ml veronal acetate buffer, pH 9.2. Levamisole (1 mg/ml) was added to the substrate to inhibit endogenous alkaline phosphatase activity (all chemicals were from Sigma, Poole, U.K.). The sections were incubated for 20–40 min in substrate, washed and mounted in Aquamount (Merck, Poole, U.K.). The bound antibodies conjugated with peroxidase were located by incubation in 0.5 mg/ml 3,4,3′,4′-tetrabiphenyl hydrochloride (Sigma, Poole, U.K.) in PBS containing 0.3% (v/v) hydrogen peroxide for 3 min. After counterstaining with Mayer's haematoxylin, the sections were dehydrated and mounted in DPX.

Control sections were taken through the same procedures, but the primary antibodies were omitted. In no instance was there any specific reaction with the control procedures.

In-situ hybridization for types I and II collagen mRNAs

(a) Preparation of riboprobes for types I and II collagens. Riboprobes for type I collagen mRNA were made from a cDNA probe, pHCAL1U. The cloned fragment of pHCAL1U is 372 bp and covers the C-terminal telopeptide and part of the C-propeptide of human proα1(I) collagen (Mäkelä *et al.*, 1988). This was subcloned into pGEM-3Z plasmid and named pGM3C1a1 (Critchlow *et al.*, 1995). To generate the antisense probe, pGM3C1a1 was linearized with HindIII and incubated with T7 polymerase; the sense probe was linearized with EcoRI and incubated with SP6 polymerase. The type II collagen riboprobe was made from the plasmid pKCol2a1-1, which carries a 400bp cDNA insert that covers the 3′-untranslated region and a small part of the C-propeptide of rabbit proα1(II) collagen mRNA (Metsaranta *et al.*, 1996). To generate the probes, pKCol2a1-1 was linearized with SmaI and incubated with T3 polymerase to give the antisense probe, or with HindIII and T7 for the sense probe. The riboprobes were labelled with digoxygenin according to the protocols provided with the kit (Roche, Lewes, U.K.).

(b) In-situ hybridization. The standard procedure (Critchlow *et al.*, 1995) included pretreatments with: (1) 0.2 N hydrochloric acid; (2) 6% hydrogen peroxide; (3) 20 µg/ml proteinase K (Sigma, EC 3.4. 21.14) in Tris-EDTA buffer, pH 8.0, for 10 min at 37°C; (4) 4% paraformaldehyde, pH 7.4 for 20 min; (5) 0.1 M glycine (2 × 10 min) and; (6) 0.25% acetic anhydride for 10 min. The sections were dehydrated through graded ethanols and 40 µl hybridization solution was applied per section. The hybridization solution (medium stringency) contained 50% formamide, 10 mM tris-HCl, pH 7.4, 1.0 mM EDTA, 0.02% polyvinyl and Ficoll, 0.02% bovine serum albumin, 0.5% sodium dodecyl sulphate, 300 mM NaCl, 10% dextran sulphate, 0.5 mg/ml yeast or *Escherichia coli* tRNA and the labelled antisense, or sense, probe, as appropriate. Hybridization was for 18 h at 55°C in a humid chamber. After hybridization, the sections were treated with 20 µg/ml RNAse in Tris-EDTA-NaCl buffer, pH 8.0. The sections were then washed twice with 1 × standard saline citrate (SSC), followed by twice with 0.5 × and twice with 0.1 × SSC; all washes were at 55°C, except the final wash which was at room temperature.

Prior to detection of the labelled digoxygenin, the sections were incubated with 0.15 M NaCl in 0.1 M Tris-HCl buffer, pH 7.5 for 5 min, followed by 0.5% Boehringer blocking reagent in the previous solution for 45 min at room temperature. The digoxygenin-label was detected using the Boehringer detection kit (Roche, Lewes U.K.). The sections were mounted in glycerine jelly.

The control hybridizations using sense riboprobes were all negative.

RESULTS

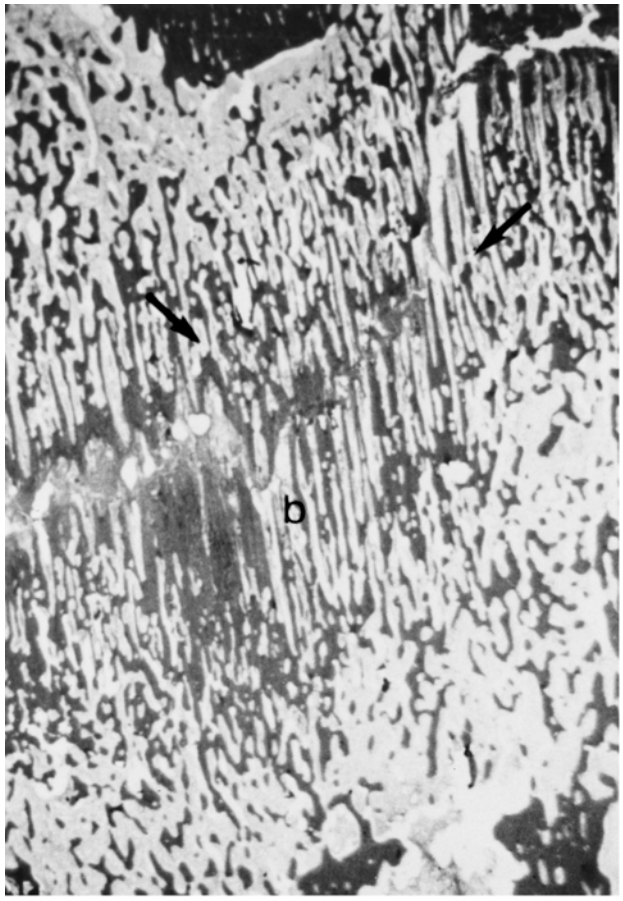
Histology of the distraction gap

The osteotomies were left in contact for 1 week before distraction was applied; during this time osteoprogenitor cells accumulated and a callus started to form. The total distraction was equal to 20% of the total length of the tibia and, therefore, the time between operation and the end of distraction varied with the distraction rate as follows—0.3 mm/day, 8 weeks; 0.7 mm/day, 4 weeks; 1.3 mm/day, 2 weeks and 2.7 mm/day, 1 week. The average increase in length of the tibiae was 20 mm.

The distraction gap in osteotomies distracted by 0.3 mm/day is filled by new bone (Fig. 1). Next to the cut ends of the cortical bone, bone remodelling has started and newly formed bone marrow cavities are present. In the central region, intramembranous bone is forming, the trabeculae of which are orientated longitudinally along the axis of the distraction force. A few areas of fibrocartilage are found in this region (Fig. 1). Microfractures induced by distraction are observed within these newly formed trabeculae (Fig. 1).

In the osteotomies distracted by 0.7 or 1.3 mm/day, new bone has grown from the ends of the cortical bone, but an area of fibrous tissue remains in the centre. Small areas of cartilage are found at the junction of the fibrous tissue and new bone, or adjacent to the periosteum (Fig. 2). Areas of necrosis and cyst formation may occur in the fibrous tissue (Fig. 3). The numbers of areas of cartilage, cysts and necrosis are greater in the 1.3 mm/day group, than in the 0.7 mm/day group.

In the group distracted by 2.7 mm/day, a small amount of bone may form around the ends of the cortical bone, particularly the proximal end. The remainder of the gap is filled by fibrous tissue, blood clots and tissue fluid (Fig. 4). Areas of cartilage similar to those found at distraction rates of 0.7 or 1.3 mm/day may be present, but they are fewer in number. Necrotic tissue is frequent.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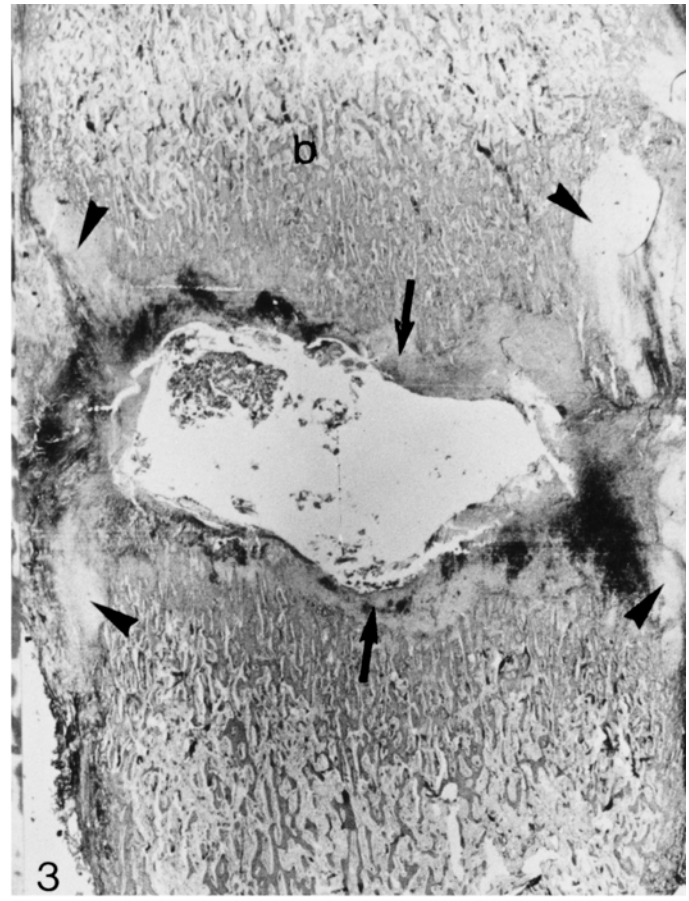
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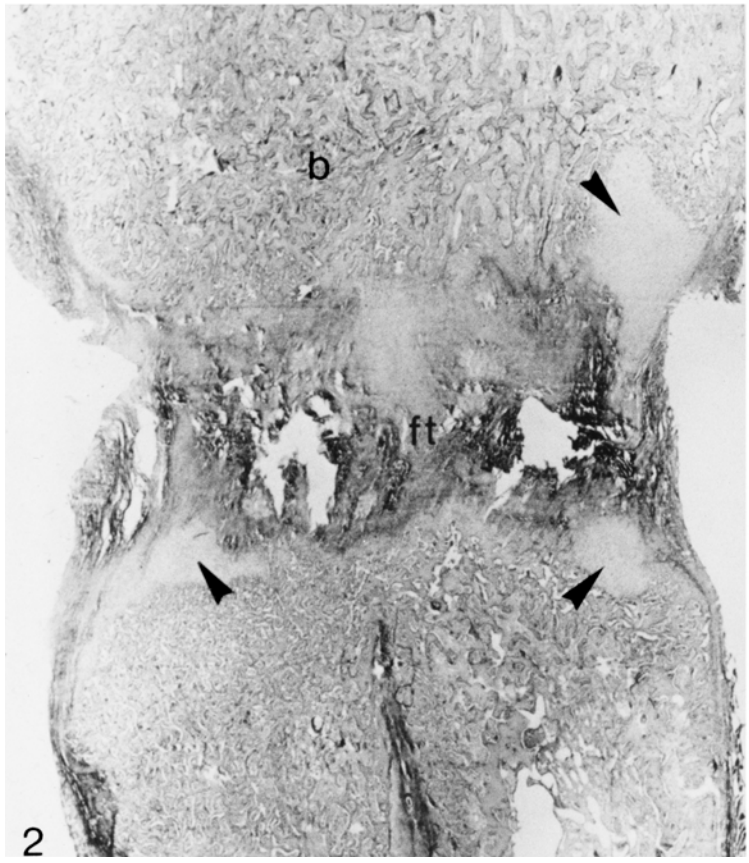
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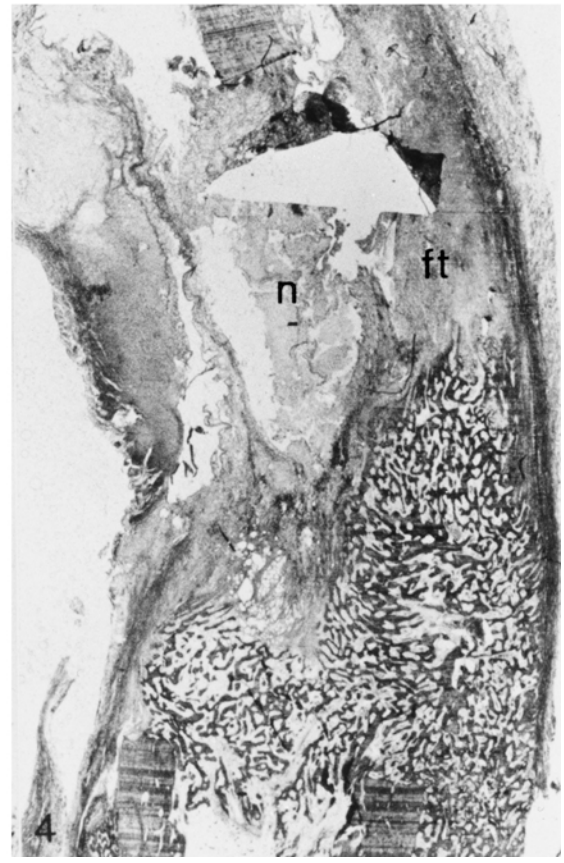
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In-situ hybridization and immunohistochemistry

In the 0.3 mm/day group, all the osteoblasts on the bone surfaces and many of the osteocytes in the newly formed trabeculae give a positive reaction for type I collagen mRNA (Fig. 5). In the central region, the cells in the fibrocartilaginous trabeculae frequently express type I and type II collagen mRNAs (Figs 5 and 6). It was not possible with the techniques presently available to determine conclusively whether the same cell expressed both mRNAs.

In the 0.7 and 1.3 mm/day distraction groups, type I collagen mRNA is localized in the osteoblasts and some osteocytes, and in the fibroblasts of the central fibrous tissue (Figs 7, 9 and 14). Where cartilage is present the boundaries between it and the fibrous tissue are not sharp. The cells in this transitional region may express both types I and II collagen mRNAs (Figs 7–10). The mature chondrocytes express only type II collagen mRNA (Figs 8 and 10). At the junctions between the bone and cartilage endochondral ossification takes place. A few hypertrophic chondrocytes still express type II collagen mRNA (Figs 8 and 10), but not type I collagen mRNA (Figs 7 and 9). In the adjacent bone, only osteoblasts and osteocytes express type I collagen mRNA (Figs 7 and 9). A similar region is shown in Figures 11–13. The cartilage matrix binds antibodies to type II collagen (Fig. 12), and the mature chondrocytes express the mRNA for type II collagen.

Figures 14 and 15 show a region in which the matrix around a small blood vessel binds antibodies to type I collagen; the surrounding tissue is cartilage-like, but still binds the antibody weakly. Type I collagen mRNA is expressed by the cells in the bone and by some chondrocyte-like cells in the cartilaginous region (Fig. 14). In the group distracted by 2.7 mm/day, a similar pattern of cells expressing mRNAs is seen as that in the 0.7 and 1.3 mm/day groups (not shown).

DISCUSSION

The progress of tissue regeneration during distraction osteogenesis is subject to three main variables: the rate, rhythm and amount of lengthening. In a clinical situation, it is essential that these are optimal. This study was designed to investigate the effect of the rate of distraction on the development of the tissue in the widening gap. The other two variables, i.e. the rhythm and total amount of lengthening (20%), were the same in all animals. As a consequence, a 20% lengthening was achieved at different time-points; for example, at a distraction rate of 0.3 mm/day in 8 weeks, whereas at 2.7 mm/day it was 1 week. Thus, the higher the rate of lengthening, the shorter the time available for tissue regeneration.

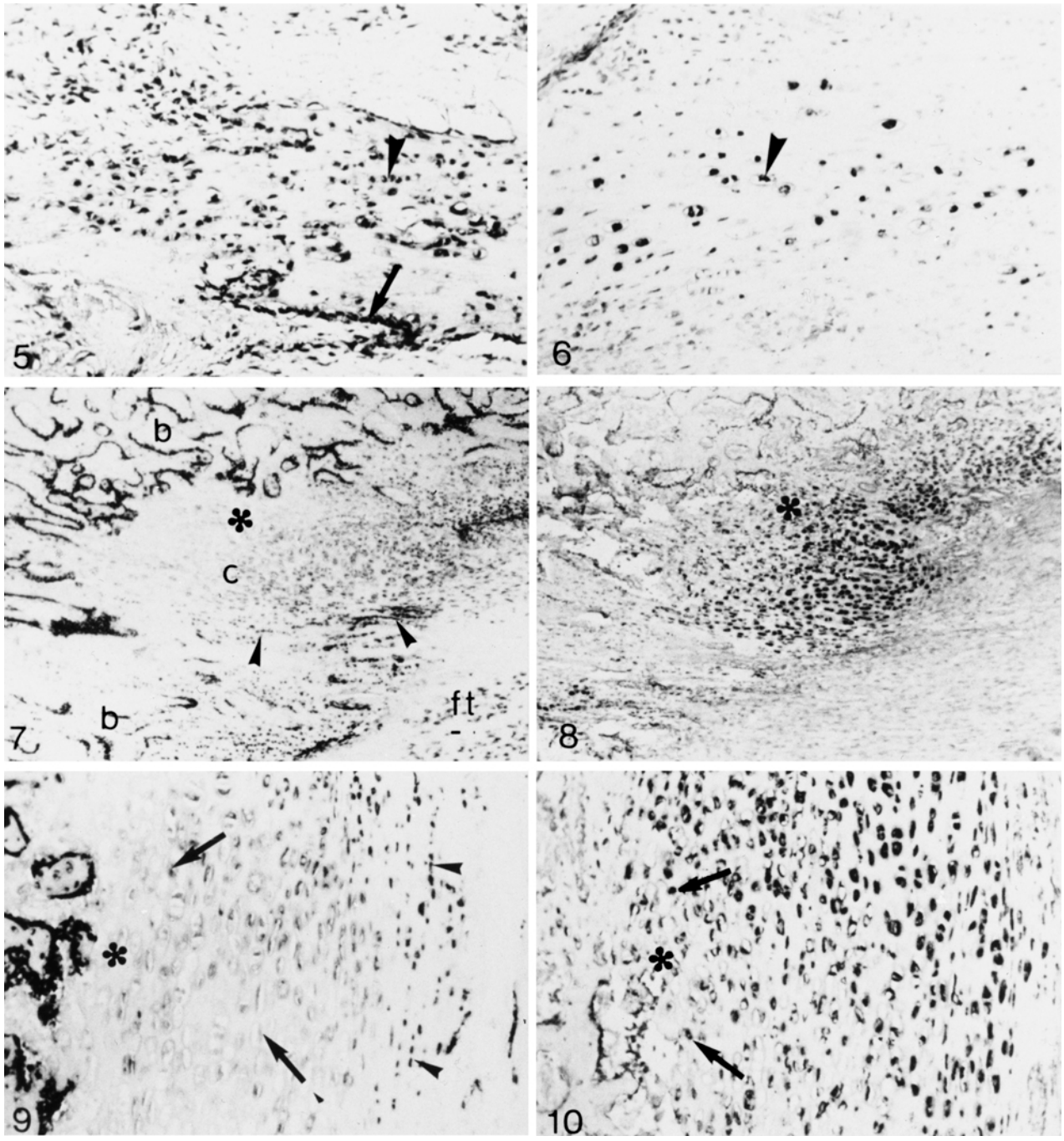
The experiment was terminated the day after 20% lengthening was achieved. Thus, no time was allowed for further bone formation and consolidation of the healing distraction gap. After distraction at 0.3 mm/day, the gap was almost entirely filled by intramembranous bone. In the centre, the trabeculae are long and there are discontinuities, or microfractures, which possibly indicate the point at which the distractive forces were pulling the tissue apart. At distraction rates of 0.7 and 1.3 mm/day, the ingrowing fronts of bone do not yet meet; an area of fibrous tissue, and at 1.3 mm/day large cysts, are found between the bone. Any cartilage is at the junctions between bone and fibrous tissue. At 2.7 mm distraction per day, the lengthening is achieved in one week, but a large area of necrotic fibrous tissue fills the centre of the gap. These observations are similar to those found after distraction osteogenesis in other animal models (Aronson *et al*., 1990; Delloye *et al*., 1990; Ilizarov, 1989a and b; Kojimoto *et al*., 1988), namely that at distraction rates of 0.5–1 mm/day, intramembranous bone is formed and fibrocartilage is

Fig. 1. At a distraction rate of 0.3 mm/day, intramembranous bone (b) is formed. Adjacent to the cut ends of the cortical bone, the bone has remodelled and marrow cavities are present. In the centre the trabeculae are longitudinal and in some regions fibrocartilage is present within them. Some micro-fractures (arrows) may be present. H & E preparation. Magnification × 6.

Fig. 2. At a distraction rate of 0.7 mm/day bone (b) has formed in the gap, but there is a central area of fibrous tissue (ft). Small regions of cartilage (arrowheads) are present between the bone and fibrous tissue. H & E preparation. Magnification × 7.

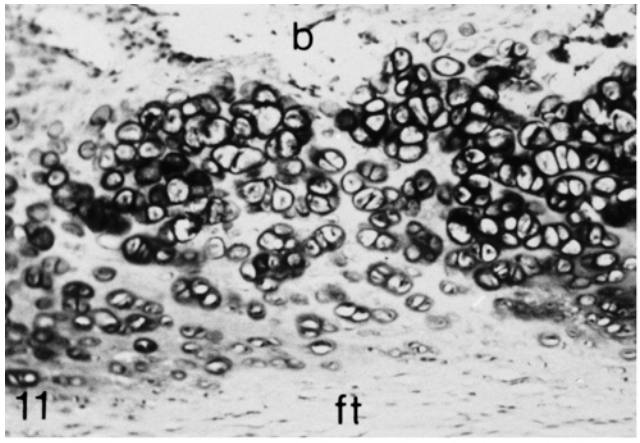
Fig. 3. At a distraction rate of 1.3 mm/day, the regenerating tissue consists of bone (b) with a central region of fibrous tissue (arrows). In this regenerate a large cyst has formed in the fibrous tissue. Small islands of cartilage are present between the bone and fibrous tissue (arrowheads). H & E preparation. Magnification × 7.

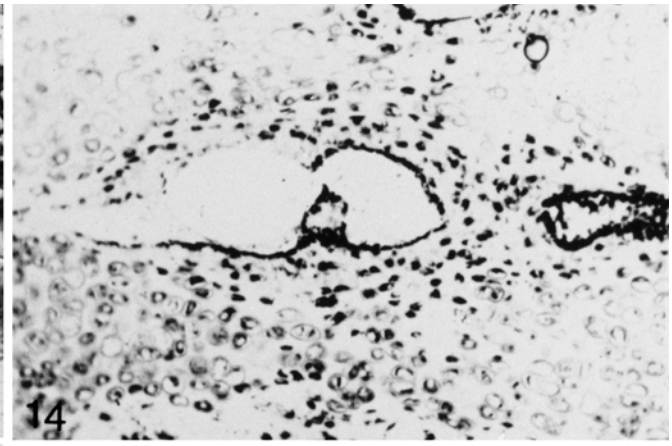
Fig. 4. At a distraction rate of 2.7 mm/day bone formation is reduced; in this regenerate it is only around one end of cortical bone. The central region is filled by irregular fibrous tissue (ft) and some necrotic tissue (n). H & E preparation. Magnification × 7.

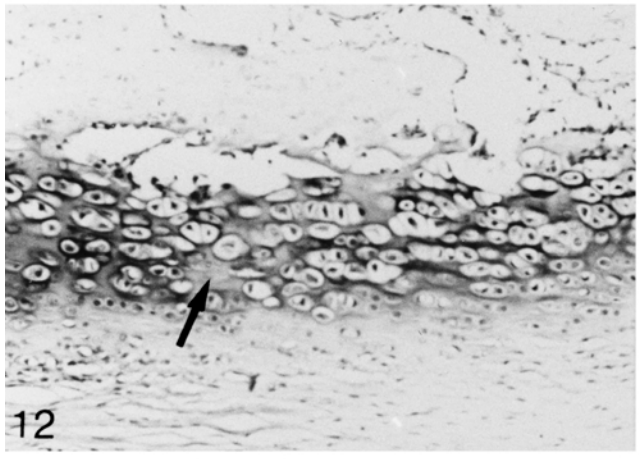


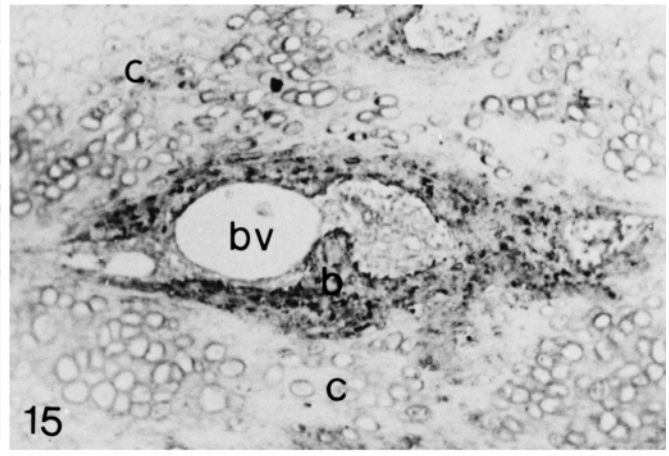
Figs 5 and 6. The central region of a regenerate distracted at a rate of 0.3 mm/day showing parts of large trabeculae in which there is some fibrocartilage. Fig. 5—*in situ* hybridization with riboprobe for type I collagen mRNA. The osteoblasts on the bone surfaces (arrow) and in the trabeculum (arrowhead) are expressing the mRNA. Fig. 6—*in situ* hybridization with a riboprobe for type II collagen mRNA. Some cells in the middle of a trabeculum are expressing this mRNA (arrowhead). Magnification of Figures 5 and 6 × 105.

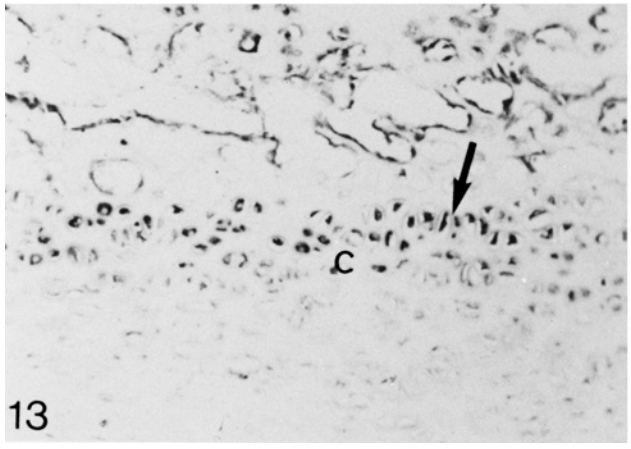
Figs 7 to 10. Adjacent sections of a small region of cartilage (c) between bone (b) and fibrous tissue (ft) in a regenerate distracted at the rate of 0.7 mm/day. The asterisks indicate approximately the same region in each Figure. Figures 7 and 9—*in situ* hybridization with riboprobe to type I collagen mRNA. The osteoblasts and some osteocytes in the bone are expresssing the mRNA. The cells in the fibrous tissue and at the periphery of the developing cartilage (arrowheads) are also expressing the mRNA. The mature and hypertrophic chondrocytes (arrows) do not express this mRNA. Figures 8 and 10—*in situ* hybridization with riboprobe to type II collagen mRNA. This mRNA is expressed by the differentiating and mature chondrocytes and some hypertrophic chondrocytes (arrows). There is some non-specific binding of the detection antibodies by the osteoblasts. Magnification of Figures 7 and 8 × 40, of Figures 9 and 10 × 105.











Figs 11 to 13. Adjacent sections of part of a regenerate distracted at the rate of 1.3 mm/day. Figure 11—H & E preparation—a narrow region of cartilage separates bone (b) from fibrous tissue (ft). Figure 12—immunohistochemical detection of type II collagen—the antibody to type II collagen is found in the cartilaginous matrix. Figure 13—*in situ* hybridization for type II collagen mRNA. The mature chondrocytes (c) are expressing this mRNA strongly. Magnification of Figures 11 to 13 × 105.

Figs 14 and 15. Adjacent sections of part of a regenerate distracted at the rate of 0.7 mm/day showing a region with a small blood vessel (bv). The blood vessel is surrounded by bone (b), but fibrocartilage (c) surrounds the bone. Figure 14—*in situ* hybridization for type I collagen mRNA—the osteoblasts in the bone and some cells in the fibrocartilage are expressing type I collagen mRNA. Figure 15—immunohistochemical detection of type I collagen—the matrix around the blood vessel binds the antibody strongly, while the fibrocartilaginous matrix binds it weakly. Magnification of Figures 14 and 15 × 105.

often present. The occasional presence of cartilage in the distraction gap was attributed to mechanical instability (Ilizarov, 1989a and b; Aronson *et al.*, 1990).

A key factor in the mechanical environment created during distraction osteogenesis is the axial stiffness of the external fixator (Waanders *et al.*, 1993). Paley *et al.* (1990) tested four external fixator systems and found that the Orthofix unilateral fixation device offers good overall biomechanical stability. This fixator was used for the present experiments.

It is obvious that local factors influencing bone formation are very important during distraction. In addition, because bone formation is dependent on a good blood supply, the rate of angiogenesis is significant. If the rate of distraction exceeds that of capillary outgrowth, an avascular central region with no bone formation is created. The distraction gaps here are $\sim$20 mm wide which means that blood vessels and bone must grow a distance of about 10 mm from the proximal and distal stumps to achieve bony union. This occurs only during distraction at the rate of 0.3 mm per day, but the lengthening took 8 weeks compared to 4 weeks or less at higher distraction rates. The lack of bone in the centre of the regenerate of the 0.7 and 1.3 mm/day groups may be attributed to the inability of capillary growth to keep up with distraction. Because all the tibiae were examined immediately after distraction ceased, it is not known how quickly the central fibrous tissue would be replaced by bone. That left after distraction at 0.7 and 1.3 mm/day should be rapidly replaced, but at 2.7 mm/day, the gap may be too large. Thus, by balancing the time required for distraction and the rate of bone formation, an optimal distraction rate for this model appears to be 0.7 mm/day.

If tissue formation in this expanding defect is compared with callus development, a number of similarities are obvious. Bone is forming at 3–5 days in a callus, but cartilage is not evident until 7 days (Ashhurst, 1986). Similar sequences apply here. There is very little cartilage at 7 days after rapid distraction, whereas there is some in those distracted for 2 and 4 weeks. The cartilage is most common at junctions between bone and fibrous tissue where mechanical stresses will occur. This is comparable with sites of cartilage in a periosteal callus, and in both instances these are areas of poor vascularity.

Collagen gene expression is affected by the distraction rate. The fibroblasts, osteoblasts and some osteocytes express type I collagen mRNA. Osteocytes do not normally express type I collagen mRNA, except in areas of rapid new bone formation, as in callus (Critchlow *et al.*, 1995). As the distraction rate rose from 0.3 to 1.3 mm/day, the number of cells expressing type II collagen mRNA and hence synthesizing a cartilaginous matrix increased. In most areas of cartilage, some cells are expressing both types I and II collagen mRNAs and the matrices contain both collagens, that is, they are areas of chondroid bone (Beresford, 1981). Such areas are common in regenerating tissues and they may have the morphological characteristics of either bone or cartilage. Type I collagen mRNA expression ceases as the chondrocytes mature, but type II collagen mRNA expression is still ongoing even in some hypertrophic chondrocytes. None of the hypertrophic chondrocytes at the endochondral ossification fronts express the type I collagen mRNA. This pattern of expression is also found in rabbit fracture callus (Bland *et al.*, 1999). There is no evidence, therefore, that the hypertrophic chondrocytes in this model transdifferentiate into osteoblasts and produce bone proteins, as suggested in other situations by Scammell and Roach (1996).

The cells in the distraction gap may derive from osteoprogenitor cells in the periosteum, marrow cavity, and surrounding tissues (Kojimoto *et al.*, 1988; Iwamoto *et al.*, 1993). Their differentiation is controlled by the local mechanical conditions and vascularity. The results in the present study indicate that differentiation of osteoprogenitor cells is affected by the distraction rate; chondrocytes are found only at rates of 0.7 and 1.3 mm/day. Irrespective of the rate of distraction, bone formation is primarily intramembranous. Endochondral ossification is restricted to those small areas of cartilage that form at the junctions of bone and fibrous tissue at the higher distraction rates.

Thus the tissues produced in the regenerate depend upon the rate of distraction. The optimal rates of distraction must favour bone formation, and the results of the present study of collagen gene expression in developing regenerates suggest that in this experimental rabbit model the optimal rate of distraction is 0.7 mm/day. Thus, It is important clinically to balance the rate of distraction with the type of tissue produced.

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