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Effect of Lengthening Rate on Angiogenesis during Distraction Osteogenesis

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Summary: This study investigated the angiogenic response to four varying rates (0.3, 0.7, 1.3, and 2.7 mm/day) of distraction in a rabbit model of leg-lengthening. Immunostaining was performed with use of specific antibodies to type-IV collagen and endothelial cell antigen to examine semiquantitatively the presence of blood vessels in the developing tissues. With use of the Chalkley counting method, the greatest number of positive-staining blood vessel cells was found in the central fibrous zone of the groups that underwent lengthening at 0.7 and 1.3 mm/day compared with any other zone in any group ($p < 0.05$, t test). There were no statistical differences in the positive labeling indices in the mineralization front and the new bone zone adjacent to the mineralization front in any of the groups. However, the decrease in the number of positive-staining blood vessel cells in the new bone zone distant to the mineralization front compared with any other zone in any group was statistically significant. The results suggest that during distraction osteogenesis, the precursor cells of new capillaries were present in abundance within the fibrous interzone. These cells connected into the capillary network at the junction of the mineralization front and the fibrous zone. The angiogenic response was weaker in the more mature regions within the new bone zones. A slow rate of distraction (0.3 mm/day) did not maximally stimulate angiogenesis in the central fibrous zone, whereas high rates (2.7 mm/day) appeared to impair this response. In this model of distraction osteogenesis, the vascularization process in the central fibrous zone was maximally stimulated at distraction rates of 0.7 and 1.3 mm/day.

The formation of new blood vessels (angiogenesis) is associated closely with osteogenesis *in vivo*. Small blood capillaries consist of two cell types: endothelial cells, resting on the basement membrane and lining the lumen, and pericytes, which are in close relationship with the endothelial cells and the capillary basement membrane. During angiogenesis *in vivo*, endothelial cells migrate from the existing vessels and, after rapid cell division, a lumen is formed and subsequent anastomosis of adjacent sprouts leads to the onset of blood flow. Bone is a highly vascularized tissue, and it has been estimated that a bone cell is no more than 200 μm away from a blood vessel (5). The vascular supply of bone consists of main nutrient arteries that are mainly from medullary arteries and the rich vasculature of the periosteum. The blood supply feeds complex sinusoidal networks within the bone and drains to venous channels, leaving through all bone surfaces not covered by articular cartilage (8).

Osteogenesis appears to be a vascular-dependent process, and angiogenesis precedes ossification during the development, repair, and regeneration of bone. The blood flow in a fracture site has been shown to reach a peak in the first 2 weeks after injury, and the bone blood flow and mineral deposition in fractured bone are closely related (8). Trueta (14) suggested that, in tibial fractures, the pattern and rate of fracture healing are dependent on the viability of the local circulation and its capacity to elicit a response.

During distraction osteogenesis, new bone is formed between the cut ends of an osteotomy as they are gradually separated mechanically. Ilizarov (7) and Aronson (1) have shown an increase in the global blood supply to the regenerating tissues during limb-lengthening. However, the neovascularization processes that occur during this procedure are still poorly defined. This study aimed to investigate these processes further by examining the effect of distraction rate on the vascularization process during distraction osteogenesis. With use of an established rabbit leg-lengthening model (9,10), the localization and distribution of two angiogenic markers, endothelial cell antigen and type-IV collagen, were studied quantitatively by immunohistochemical methods.

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MATERIALS AND METHODS

Animal Model and Sample Preparation

Sixteen adult New Zealand White rabbits (age: 24 weeks; body weight: 3.5-4.0 kg) were randomly divided into four groups, and a

FIG. 1. Type-IV collagen immunostaining (arrows) in the regenerating tissues of the 0.3 mm/day distraction group was generally weak. View showing new bone zone (NB) and central fibrous zone (F) (magnification, ×25). The mineralization front was very narrow and barely distinguishable at this distraction rate.

mid-tibial osteotomy was performed in the left tibia of each animal. The tibiae were stabilised with external fixators as previously described (9,10). Seven days later, distraction was initiated twice daily at rates of 0.3, 0.7, 1.3, and 2.7 mm/day, until the tibiae were lengthened to 20% of the original length (approximately 2 cm). As soon as the appropriate lengthening had been achieved, the animals were killed and the regenerating tissues of the distraction gaps were collected. Specimens were fixed in 4% paraformaldehyde (pH 7.4) for 24 hours before decalcification in buffered 14.5% EDTA (pH 7.2) for 3-4 weeks at 4°C. Decalcification was confirmed by radiography, and the decalcified specimens were embedded in paraffin wax. Sections, 5 μm thick, were cut with use of a microtome and placed on poly-L-lysine (Sigma-Aldrich, Poole, England)-coated slides for histology and immunohistochemistry. For routine histological examination, the sections were stained with haematoxylin and eosin.

Immunohistochemistry with Antibodies to Type-IV Collagen and Endothelial Cell Antigen

Immunohistochemistry using specific antibodies to type-IV collagen and endothelial cell antigen (CD 31) was performed by an indirect two-step method: 5-μm-thick paraffin sections were dewaxed in xylene and taken through graded ethanol (100, 90, 70, and 50%). Endogenous alkaline phosphatase activity was blocked by immersion of the sections in Tris buffered saline containing 0.1 m*M* levamisole (Sigma-Aldrich) for 30 minutes at room temperature. The sections were then enzymatically treated with protease type XXIV (0.125 mg/ml; Sigma-Aldrich) for 20 minutes at 37°C. Following washes in Tris buffered saline, they were incubated with monoclonal antibodies, either mouse anti-human type-IV collagen (code: M0785; DAKO, Cambridge, England) at a dilution of 1:40 or mouse anti-human endothelial cell antigen (CD 31, code: M0719; DAKO) at a dilution of 1:50, for 1 hour in a humid chamber at room temperature. After three rinses in Tris buffered saline, the sections were incubated with a rabbit anti-mouse alkaline phosphatase-conjugated antibody (Sigma-Aldrich) at a dilution of 1:50 in Tris buffered saline for 30 minutes. After incubation, the sections were washed in Tris buffered saline and treated with a visualization solution containing substrates of 10 ml Tris buffered saline, 400 μl naphthol AS-MX buffer (Sigma-Aldrich), and 10 mg fast red (BDH Chemicals, Dorset, England) for 30 minutes in darkness. The slides were rinsed in running water, counterstained with Mayer's haematoxylin for 3 minutes, dehydrated through graded ethanols, cleared in xylene, and finally mounted with Aquamount (BDH Chemicals). As a negative control, normal rabbit serum was used in the procedure instead of the primary antibodies. For the positive control, tissues known to contain type-IV collagen or endothelial cell antigen, including human blood vessels and skin, were used. All stained sections were examined and photographed with use of a photomicroscope (Axiophot; Zeiss Instruments, Oberköchen, Germany).

Quantitative Assessment of the Immunostaining Results by the Chalkley Counting Method

To quantitate the immunostaining results, the Chalkley count-

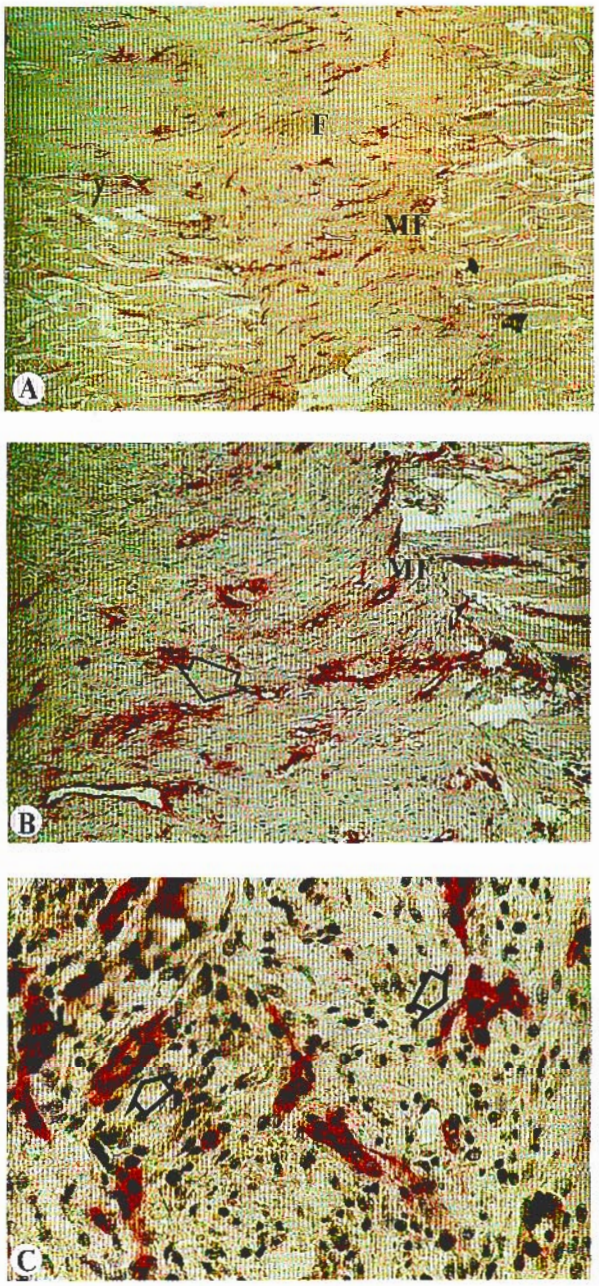

FIG. 2. A: Compared with the 0.3 mm/day group (Fig. 1), immunostaining by type-IV collagen antibody showed increased binding in the fibrous zone (F) and the mineralization front (MF) in the regenerating tissues of the 0.7 mm/day group (magnification, ×25). **B:** In the mineralization front, the new blood vessels immunostained by type-IV collagen antibody along with the new bone formed parallel to the direction of the distraction (arrow) (magnification, ×100). **C:** The outline of the new vessel buds by endothelial cell antigen immunostaining in the fibrous region (arrows) (magnification, ×100).

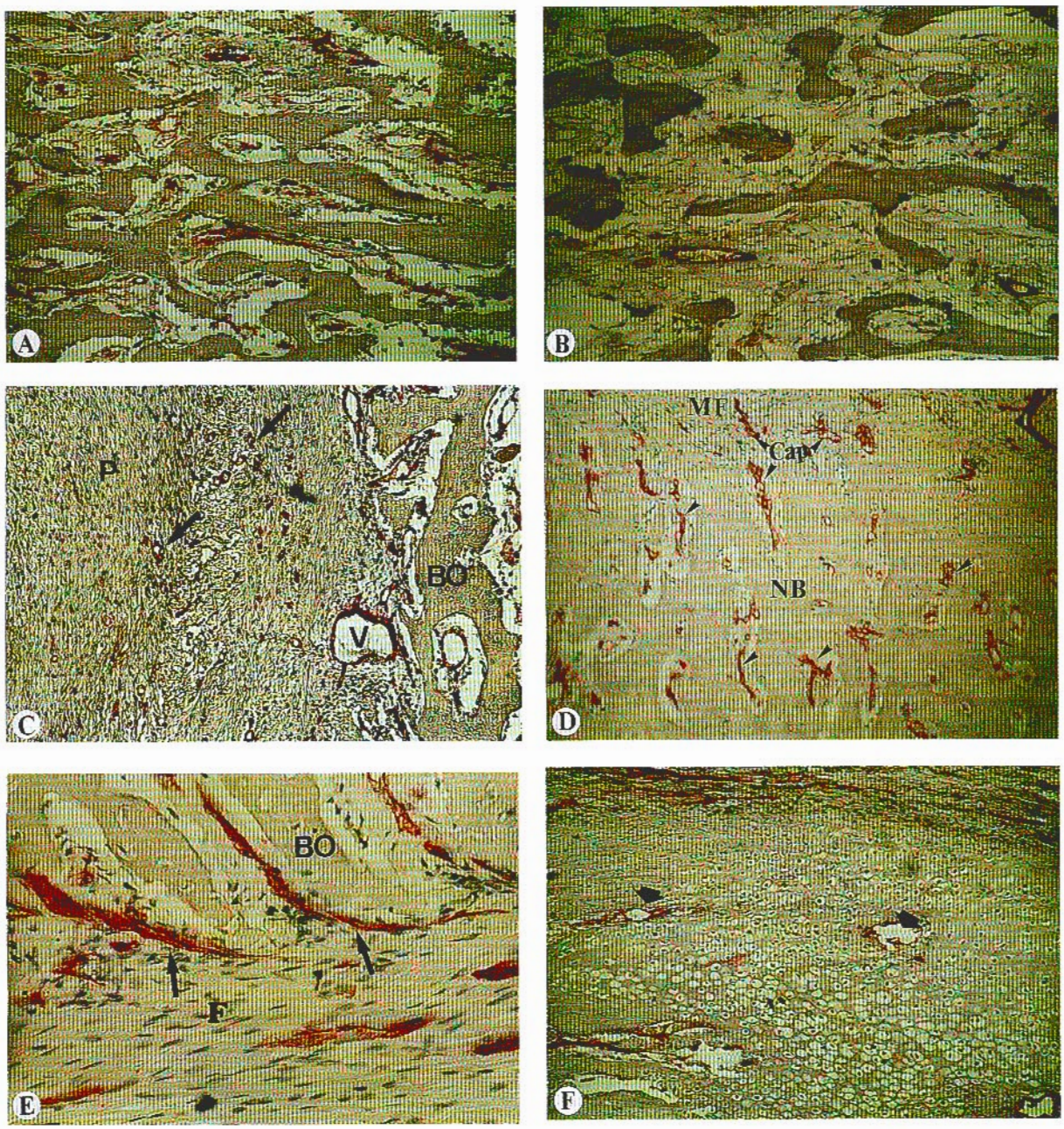

FIG. 3. A: The staining of type-IV collagen in the new bone zone adjacent to the mineralization front was strong (magnification, ×100). **B:** The staining of type-IV collagen became weaker in the new bone zone distant to the mineralization front (magnification, ×100). **C:** Staining of the blood vessel (arrows) in the surrounding soft tissues adjacent to the regenerated area in the 0.7 mm/day group was strong. Endothelial cell antigen immunostaining (magnification, ×100). P = periosteum, V = vessel, and BO = bone. **D:** In the 0.7 mm/day group, new bone (NB) formed in close contact with the new capillaries (Cap) (arrows). Type-IV collagen immunostaining (magnification, ×100). MF = mineralization front. **E:** Intramembranous bone (BO) formation was observed close to the blood vessels in the fibrous tissue (F). Type-IV collagen immunostaining (magnification, ×400). **F:** Positive-staining cells were absent in the regions of cartilage and were present only in the vicinity of rare invading blood vessels (arrows). Type-IV collagen immunostaining (magnification, ×100). All immunostained sections were counterstained with Mayer's haematoxylin.

ing method, an established and validated method of quantitating angiogenesis (4,6), was used. Briefly, the area containing the maximum number of microvessels was first chosen by scanning the immunostained section at low power (×40). Any immunoreactive endothelial cells that were separate from adjacent capillaries were counted as vessels. A 25-point Chalkley point eyepiece graticule was employed over the selected region and oriented so that the maximum number of points at ×250 (0.155 mm^2) was on or within areas of highlighted vessels. The mean of the three Chalkley counts of each morphologically defined zone was generated for each section. This was repeated twice, and the data were then analysed with use of one-way analysis of variance and Student's t test.

RESULTS

Immunostaining Results of Type-IV Collagen and Endothelial Cell Antigen

Staining for type-IV collagen and endothelial cell antigen was evaluated at four separate regions of the

FIG. 4. The immunostaining for endothelial cell antigen (arrows) was greatly reduced in the central fibrous zone of the regenerated area in the 2.7 mm/day group. Endothelial cell antigen immunostaining (magnification, ×25).

regenerated area, which have been defined previously (10,11): (a) the central fibrous region, (b) the mineralization front, (c) the new bone zone adjacent to the mineralization front, and (d) the new bone zone distant to the mineralization front. In the 0.3 mm/day group, the distraction gap was almost filled with newly formed bone tissue, with only a narrow central zone of fibrous tissue. Staining for type-IV collagen (Fig. 1) and endothelial cell antigen (not shown) was present throughout the regenerating tissues but was weak in the new bone zone.

The immunostaining patterns for type-IV collagen and endothelial cell antigen were very similar in the 0.7 and 1.3 mm/day groups (Fig. 2). However, compared with the 0.3 mm/day group, the central fibrous zone was larger and there was significantly more staining for both type-IV collagen (Fig. 2A and B) and endothelial cell antigen in the mineralization front and the central fibrous zone. Intense staining for type-IV collagen (Fig. 2A and B) and endothelial cell antigen (Fig. 2C) showed that new blood vessels formed parallel to the direction of distraction. The morphology of the endothelial cells in the fibrous region was similar to that of the fibroblasts; therefore, only by immunostaining with use of antibody to endothelial cell antigen was it possible to distinguish the endothelial cells from the fibroblasts. The formation of new vessel buds was also clearly outlined by staining of endothelial cell antigen (Fig. 2C). Staining of type-IV collagen and endothelial cell antigen was evident throughout the new bone zone but tended to be reduced toward the original bone ends (Fig. 3A and B). The staining was sparse or absent in the region of intact cortical bone. The significant contribution of the surrounding soft tissues (periosteum and muscle) to the neovascularization of the regenerating callus was observed in the 0.7 and 1.3 mm/day groups. New vessel formation was enhanced in the surrounding thickened periosteum (Fig. 3C) and nearby skeletal muscle (not shown) in the 0.7 and 1.3 mm/day groups. In the new bone zone, newly forming bone and blood vessels were in close contact and the new bone formed only where blood vessels lay (Fig. 3D). New bone formed directly from the periosteum in proximity to the new blood vessels (Fig. 3E). Cartilage islands were found in the regenerated areas of groups with distraction rates higher than 0.7 mm/day. Staining for type-IV collagen and endothelial cell antigen was absent in the bulk of the cartilage region and was present only in the vicinity of rare invading blood vessels (Fig. 3F).

At a distraction rate of 2.7 mm/day, most of the

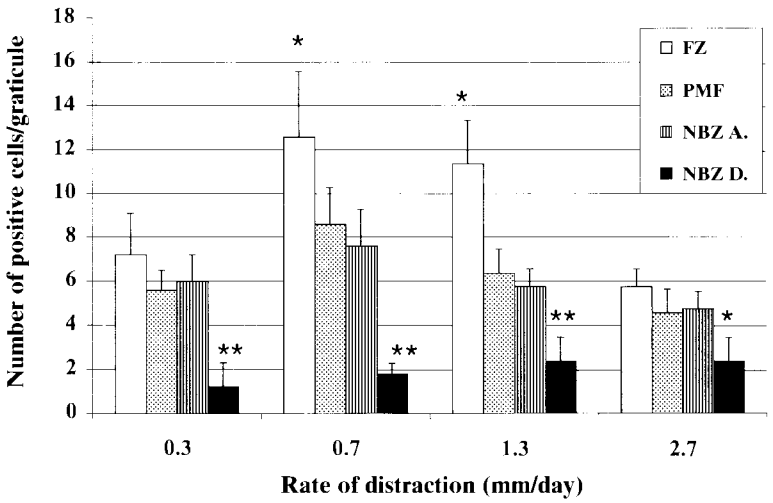

FIG. 5. The Chalkley counting results showed that the number of blood vessels is statistically higher in the central fibrous zone (FZ) of the 0.7 and 1.3 mm/day groups compared with any other zone of any group (*$p < 0.05$, t test). There are no statistical differences in the number of positive-staining blood vessel cells in the primary mineralization front (PMF) or the new bone zone adjacent to the primary mineralization front (NBZ A.). The number of positive-staining cells in the new bone zone distant to the primary mineralization front (NBZ D.) is significantly decreased compared with elsewhere in the regenerated area in any group (*$p < 0.05$; **$p < 0.01$, t test). Means and SDs are plotted.

distraction gap was filled with fibrous tissue, with only sparse bone formation. Both type-IV collagen (not shown) and endothelial cell staining (Fig. 4) appeared to be greatly reduced in the central fibrous zone, the mineralization front, and the new bone zone compared with similar regions in the 0.7 or 1.3 mm/day groups. The negative controls, in which the primary antibodies for both type-IV collagen and endothelial cell antigen were omitted, had no staining (not shown).

Semiquantitative Assessment of Blood Vessel Number by Chalkley Counting

With use of the Chalkley counting technique, the number of blood vessels was found to be significantly higher in the central fibrous zone ($p < 0.05$, t test) in the 0.7 and 1.3 mm/day groups compared with the number of positive-staining cells in any other zone of any group (Fig. 5). The number of positive-staining cells in the mineralization front or new bone zone adjacent to the mineralization front was not statistically different from each other in any of the groups. However, the number of blood vessels was reduced statistically in the new bone zone distant to the mineralization front compared with the fibrous zone, the mineralization front, and the new bone zone adjacent to the mineralization front in the 0.3, 0.7, and 1.3 mm/day groups ($p < 0.01$, t test) and the 2.7 mm/day group ($p < 0.05$, t test, Fig. 5). The angiogenic response appeared to be reduced from the mineralization front to the new bone zone adjacent to the mineralization front in all groups, and this reduction became statistically significant in the new bone zone distant to the mineralization front in all groups compared with any other zones within the groups ($p < 0.01$ in the 0.3, 0.7, and 1.3 mm/day groups, and $p < 0.05$ in the 2.7 mm/day group, t test, Fig. 5).

DISCUSSION

Angiogenesis is a complex multistep process involving extracellular matrix remodelling, endothelial cell migration and proliferation, capillary differentiation, and anastomosis. Angiogenesis is tightly regulated and is observed only transiently in particular circumstances such as tissue development, wound healing, and fracture repair. The early vascular response is essential for the normal progress of fracture healing. Chidgey et al. (2) have demonstrated a direct and predictable relationship between the phase of vascular reorganization and the rigidity of fracture healing. Using quantitative technetium scintigraphy, Aronson (1) has shown that blood flow increases to almost 10 times control levels during distraction osteogenesis after osteotomy, peaks at 2 weeks postoperatively, and decreases to four to five times the control levels for the remainder of the distraction period. During the consolidation period, an increased flow persists at levels of two to three times that of the control. These observations are confirmed by findings in the present study that the fibrous interzone and mineralization front have the most blood vessel-forming cells and that there is a marked decrease in numbers of positive-staining blood vessel cells in the mature region of the new bone zone. Aronson also found that each column of new bone was completely surrounded by large vascular sinusoids, with osteoblasts at the tip or on the surfaces of each column in close relation to these sinusoids. The results in the present study using angiogenic immunohistochemical markers to identify blood vessel cells concur with these previous findings. In agreement with the reports by Ilizarov (7) and Aronson (1), an enhanced angiogenic process was observed during distraction osteogenesis at optimal distraction rates (0.7 and 1.3 mm/day), which have been defined previously in a similar animal model (9,10), whereas a slow distraction rate (0.3 mm/day) did not maximize the angiogenic potential of the tissue and an excessive rate of distraction (2.7 mm/day) inhibited or reduced angiogenesis in the regenerating tissues.

In the present study, we used four different rates to investigate the effects of distraction rate on the angiogenic response of regenerating tissue. The amount of lengthening is kept constant and the distraction rate is varied; therefore, it is inevitable that the tissue specimens are obtained at different times after the osteotomy. For all groups, a latency period of 7 days follows the osteotomy to allow recovery of soft tissue and blood supply, and lengthening is applied thereafter at a consistent rate in a standard manner. It is therefore reasonable to assume that the response of the tissue in the distraction gap to the lengthening is consistent throughout the lengthening period. However, it is also reasonable to argue that the different times of sampling may reflect the different stages of the angiogenic process, and it should be borne in mind that the current study is based on a one-time window only, so that the dynamic changes of angiogenic processes at four different rates cannot be examined by the present study design. The current study concentrates only on the state of the regenerating tissue at the end of distraction at different rates; the regenerating tissues were harvested immediately after the 20% lengthening was achieved. The distraction was applied continuously in all groups for the experimental periods; therefore, it is acceptable to compare the cellular response to various distraction rates in the regenerating tissues at the end of distraction. We have used the same experimental design while studying the effect of distraction rates on cell proliferation during distraction osteogenesis and found that, of the four distraction rates, 0.7 mm/day was the most satisfactory for promoting bone-forming cell proliferation (9,10).

Taken together, the results suggest that distraction at 0.7 mm/day was most satisfactory for both osteogenesis and angiogenesis in this experimental system of leg-lengthening. Higher rates (2.7 mm/day) create the appropriate lengthening gap in less time, and the large increment of each distraction damages the regenerating tissues; this appears to inhibit the angiogenic process at the center of the regenerating tissue.

In the 0.7 and 1.3 mm/day groups, the intense staining of both type-IV collagen and endothelial cell antigen was localised mainly in the central fibrous zone and the mineralization front. However, previous work (1) has indicated that the fibrous interzone has a relatively decreased blood flow, whereas the adjacent zones of new bone formation, including even the host bone surfaces, have marked increases in blood flow. The present study is based on examination of the distribution of the blood vessel-forming cells using immunohistochemical methods, and the positive-staining cells do not necessarily represent the existing anastomotic network. The immunostaining results have shown that the capillary precursor cells and their early matrix formation are present in abundance within the fibrous interzone. These elements may not yet be connected into the existing capillary networks until reaching the junction between the bone mineralization front and the fibrous zone. These results are consistent with findings with a vascular injection technique that the blood flow appears to come from both proximal and distal sites of the bone toward the center of the regenerated area (1). In addition, the most intense stainings for cell proliferation and bone morphogenetic protein (BMP)-4 gene expression were observed in the mineralization front and the fibrous zone in our previous studies (10,11). These combined results support the suggestion that endothelial cells play an important role in differentiation and proliferation of osteogenic cells, by either endothelial cells or pericytes providing a supplementary source of osteoblasts and thereby participating directly in the process of intramembranous bone formation (3). Alternatively, as suggested by Schor et al. (13), pericytes and endothelial cells of blood vessels may act as links between angiogenesis and mineralization. A further role for angiogenesis in ossification is indicated by Reddi and Cunningham (12), who reported that native BMP-3 and recombinant BMP-4 bind to type-IV collagen of the basement membrane, and this novel interaction indicates that angiogenesis and vascular invasion may be a prerequisite for bone morphogenesis.

In conclusion, the present study indicates that the angiogenic process was affected by the rate of distraction; a slow rate (0.3 mm/day) did not maximally stimulate the angiogenic response whereas higher rates (2.7 mm/day) inhibited angiogenesis. In the present animal model of distraction osteogenesis, the angiogenic process was maximally enhanced by distraction stimuli at rates of 0.7 and 1.3 mm/day.

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