Severe hindlimb ischemia causes periosteal proliferation in the rat tibia

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In this rat study, we found tibial periosteal hyperplasia and hypertrophy, and appositional new bone formation 3 days after transient hindlimb ischemia. This response was positively correlated to the extent of muscle necrosis, which was increased either by raising the environmental temperature during ischemia or by prolonging the period of ischemia.

By changing the temperature from 21 °C to 34 °C, the area periost in percent of the total tibial area was increased from 5 to 17, and by changing the duration of ischemia from 3 to 5 hours, it increased from 8 to 18.

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Local tissue trauma such as bending, compression, fracture, interruption of blood vessels and hematoma formation induce periosteum, endosteum and bone to produce callus and new bone (Hulth 1990, Göransson et al. 1992, Raab-Cullen et al. 1994, Torrance et al. 1994). Signal substances which possibly mediate these reactions are prostaglandins, bone morphogenetic protein, interleukins, tumor necrosis factor, growth factors and endothelin (Hulth 1990, Battistini et al. 1993). These factors are released after various types of local trauma in which ischemia may be a common factor. We have previously reported that transient ischemia stimulates periosteal hypertrophy and hyperplasia in the rat tibia (Svindland et al. 1995), and we now investigate whether this phenomenon depends on the severity of the ischemic insult.

Animals and methods

Induction of ischemia

Male Wistar/Han/Mol SPF rats, 8 weeks old, weighing 250–300g, were anesthetized with a combination of fentanyl 0.05 mg/mL, fluanison 2.5 mg/mL, and midazolam 1.25 mg/mL. Initially, 2.3 mL/kg was given subcutaneously, followed by 1.7 mL/kg per hour, given intraperitoneally during the period of ischemia and early reperfusion. Buprenorphine 0.2 mg/kg was given for pain relief postoperatively. The experiments conformed to the Norwegian Council of Animal Research Code for the Care and Use of Animals for Experimental Purposes.

The femoral vein, artery and nerve were dissected free, distal to the inguinal ligament in the left leg. The sciatic nerve was dissected free through a separate incision proximal on the thigh. Two silicone tubes were pulled through the thigh just medial to the femur and used as tourniquets to occlude all collateral vessels, leaving the main nerves and vessels free. The femoral artery was then occluded with a microvascular clip. Previous studies have shown that this technique causes complete circulatory arrest in the lower leg (Skjeldal et al. 1991).

The animals were placed in an incubator during ischemia and the first 2 hours of reperfusion. The tourniquets and clamps were released after a predesignated period of ischemia, 0.5% lidocain chloride was applied locally to prevent spasms in the vessels during early reperfusion, and pulses in the femoral artery were checked distal to the occlusion site, before the wounds were closed. The animals were given 5 mL of 0.9% NaCl intraperitoneally at the end of the ischemic period to prevent dehydration.

72 hours after the end of the ischemic period, the rats were anesthetized, the left leg was exarticulated in the hip and the animals were killed with an overdose of pentobarbital. The skin was removed and the whole leg was fixed in 4% phosphate-buffered formaldehyde and later decalcified in 30% hydrochloric acid for 24 hours. Tissue blocks from the middle part of the lower legs were embedded in paraffin and cross-sections were cut at 4 µm and stained with hematoxylin and eosin.

Quantification of muscle necrosis

The areas of necrosis in the anterior tibial muscles were measured by morphometry, as described previously (Skjeldal et al. 1991).

Quantification of periosteal proliferation

The periosteum was defined as an outer fibrous and an inner osteogenic layer (Ham and Harris 1971). The inner layer (cambium) of osteoprogenitor cells was identified only in sections with proliferation of periosteum, while the outer fibrous layer was found in all sections. A periosteal area consisting of both these layers was measured and presented in percentage of the total cross-sectional area of tibia, including periosteum, cortical bone and medullary area (Svindland et al. 1995).

Experimental groups

Temperature study. 24 animals were randomized into 4 equal groups, 6 in each. Complete ischemia was maintained for 4.5 hours. The incubator temperature during ischemia was 21°, 24°, 27° and 34 °C in the 4 groups respectively. The results concerning necrosis in the anterior tibial muscle (Skjeldal et al. 1992) and the periosteal changes in the 27 °C group (Svindland et al. 1995) have been published previously. Reexamination of the changes in bone and periost in all groups from this series adds new information and we present this series together with the time study.

Time study. 56 animals were randomized into 7 groups, 8 in each. Complete ischemia was maintained for 0 (sham operation), 3.0, 3.25, 3.5, 4.0, 4.5 and 5.0 hours in each group respectively. The incubator temperature was kept at 27 °C during ischemia and early reperfusion in all groups.

Statistics

The periosteal areas of the different groups in each study were compared, using analysis of variance (ANOVA). $P < 0.05$ was considered significant. Simple regression analysis was used to investigate the relation between periosteal area and temperature and duration of ischemia in each group, and the area of muscle necrosis in both studies pooled.

Results

All animals, except those in the sham group, had paresis in the operated limb. 5 animals died during the postoperative period, and 1 case was excluded due to technical problems with the preparation, leaving 21 animals for analysis in the temperature study and 53 animals for analysis in the time study. All 74 cross-sections had a well defined tibia, periosteum and anterior tibial muscle (Figure 1). Muscle necrosis was found in all sections, except in 3 animals in the 21 °C group (temperature study). Regardless of the duration of ischemia or the ambient temperature, the core of the anterior tibial muscle was most vulnerable (Figure 1). In cases of severe ischemia, the central parts of the lesions showed pan-necrosis with no signs of reflow, while the peripheral parts showed partial destruction of the fibers. Both zones increased in size with increasing periods of ischemia or with increasing ambient temperature. In the temperature study, the total area of muscle necrosis increased from 9% (mean) in the 21 °C group to 100% in the 34 °C group. In the time study, it increased from 83% (3.0 hours ischemia) to 98% (5.0 hours of ischemia).

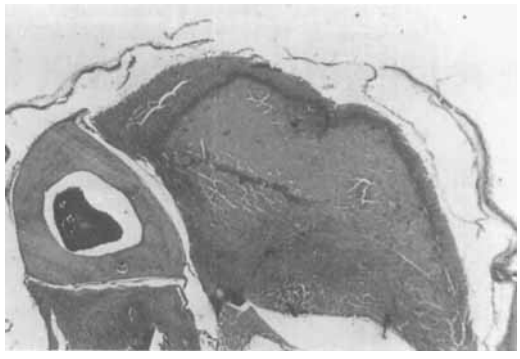
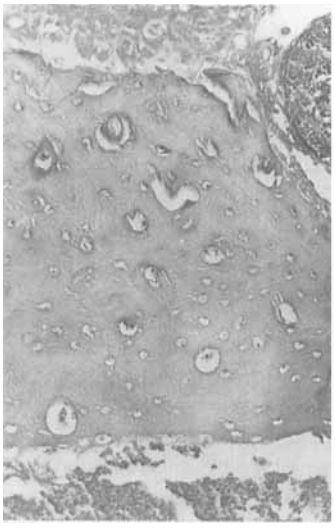


Figure 1. Cross-section of the middle part of the lower leg from an animal subjected to 3.25 hours of ischemia. Clearly delineated tibia and anterior tibial muscle with necrosis in the core of the muscle (HE, ×13). Periosteum is not discernible at this magnification.

3 animals subjected to 5 hours of ischemia (time study) had necrotic bone marrow, but normal cortical bone and proliferating periosteum. The animals exposed to 34 °C (temperature study) had granulocytes and macrophages in the medullary area and pycknotic osteocytes or empty lacunae in the cortical bone.

The other animals had a well-defined laminated bone, osteocytes in most lacunae and a normal bone marrow (Figure 2B). The marrow cavity was lined with a single layer of cuboid cells forming the endosteum and showed only sparse proliferation in the most affected animals.

In the sham-operated animals, the osteogenic layer of periosteum was not distinguishable, but in the groups with most extensive muscle necrosis this part was clearly visible and proliferated up to more than 20 cell-layers (Figure 3). Differentiation into osteoblasts and formation of new immature, woven bone was visible in this area, as shown in Figure 3. In the temperature study, there was a positive correlation between areas of periosteum and temperature ($r = 0.78$).



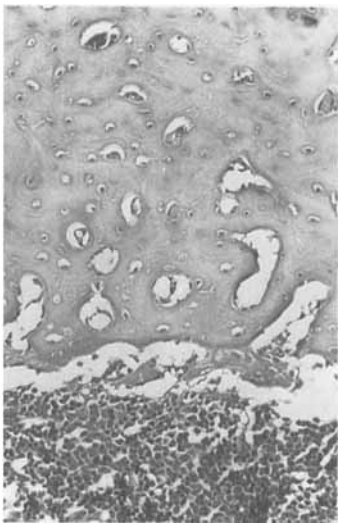
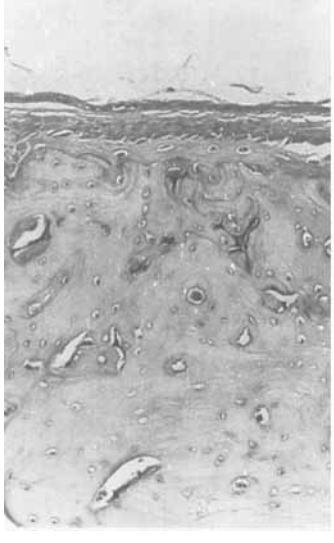


Figure 2. A. Cross-section from a rat subjected to 4.5 hours of ischemia at an environmental temperature of 34 °C. Necrotic bone marrow (at the bottom) and empty lacunae in the cortical bone (HE, × 200).

B. Normal bone and bone marrow from animal subjected to 3 hours of ischemia (HE, ×200).



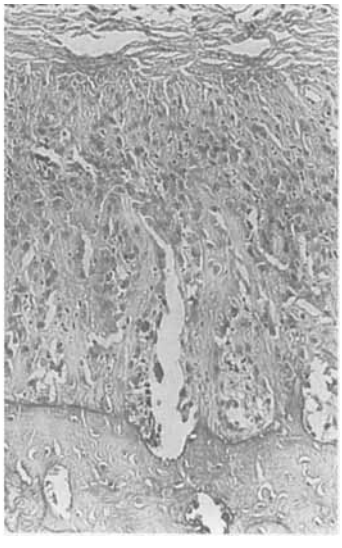


Figure 3. A. Cross-section of the tibia from animal subjected to 3.25 hours of ischemia, showing moderate periosteal response (HE, ×200).

B. Cross-section of the tibia (4.5 hours of ischemia) demonstrating marked response (HE, ×200).

In the most damaged legs (34 °C), this induction seemed to have been aborted, as the periosteum was thickened but necrotic (no viable cells), and there was no formation of new bone (Figure 2A).

The time study showed a significant increase from 8% periosteal area after 3.0 hours of ischemia to 18% after 4.5 hours ($r = 0.69$). ANOVA showed significant differences between the groups (Tables 1 and 2) in both studies ($p < 0.001$). When analyzing both studies together, area muscle necrosis correlated with area periost ($r = 0.52$, $p < 0.0001$), and induction of periosteal proliferation seemed to require an ischemic insult that caused at least 80% muscle necrosis.

Discussion

The main findings were a pronounced tibial periosteal hyperplasia, hypertrophy, and appositional new bone formation 3 days after transient hindlimb ischemia in rats. This response was positively correlated to the extent of muscle necrosis which was increased either by raising the environmental temperature during ischemia or by prolonging the period of ischemia.

In animals having complete muscle necrosis without reflow, these reactions seemed to have been aborted. According to the data from the temperature and the time studies there had to be extensive ischemia for the periosteal reaction to occur. Previous studies have shown that after shorter periods of ischemia there was a reactive hyperemia in the anterior tibial muscle, but occlusion for 4.5 hours caused a long postischemic period of hypoperfusion and large areas of no reflow (Skjeldal et al. 1993).

The surgical dissection and the application of tourniquets in the groin caused no reaction in the tibia, as there was a normal lining of fibrous periosteum in the sham-operated animals. There was no proliferation in the 21 °C group. We therefore conclude that the ischemia per se, and not the surgical trauma, was the stimulus. The contralateral leg was not used as control, which might have been of interest in seeking a possible effect of circulating mitogenic agents, but a later study (unpublished) showed no such systemic reactions.

The cambial layer contains osteoprogenitor cells

Table 1. Area periost as percentage of the total cross-sectional tibial area of rats after 4.5 hours of hindlimb ischemia at different environmental temperatures and 3 days' survival. ANOVA showed an overall significance level ($p < 0.001$). Regression analysis showed a positive correlation ($r = 0.78$)

Temperature during ischemia, °C	Number of animals	Area periost in % mean	SD
21	6	6	1.1
24	6	11	2.2
27	6	17	4.7
34	4	18	7.0

Table 2. Area periost as percentage of the total cross-sectional tibial area of rats after transient hindlimb ischemia of different durations and 3 days' survival. ANOVA showed an overall significance level ($p < 0.001$). Regression analysis showed a positive correlation ($r = 0.69$)

Duration of ischemia (h)	Number of animals	Area periost in % mean	SD
0	5	4.9	1.5
3	8	8.7	2.9
3.25	8	9.7	4.1
3.5	8	13	7.3
4.0	7	13	3.3
4.5	7	18	2.5
5	8	17	5.1

(Cornell and Lane 1992). Transforming growth factor-ß injected subperiosteally in young rabbits has been shown to increase the proliferative rate of these cells, but not to initiate osteoblastic or chondrocytic differentiation (Critchlow et al. 1995). It also caused a breakdown of the fibrous layer, a phenomenon not found in our series. A number of other growth factors, released after tissue trauma, probably stimulate determined osteoprogenitor cells during early fracture repair. The vasoactive peptide endothelin activates these factors, acting in synergism with them, they may, in turn, stimulate release of more endothelin, which thus in a paracrine or autocrine manner mediates vascular remodeling and mitogenesis (Battistini et al. 1993).

Early removal of fracture hematoma has been shown to reduce periosteal callus production, and late removal impaired the healing even more (Grundnes and Reikerås 1993b). This may be due to a reduced amount of cytokines and growth factors. Oxygen-derived free radicals released by leukocytes during reperfusion seem, however, to impair fracture repair (Göktürk et al. 1995). Reaming before nailing of fractures or osteotomies in long bones has been found to cause an acute ischemia in cortical bone and periosteum (Grundnes and Reikerås 1993a, Schemitsch et al. 1994).

On the contrary, Reichert et al. (1995) found an increase in periosteal flow 30 minutes after reaming of tibia in sheep. They ascribe this phenomenon to reversal of flow from a centrifugal to a centripetal direction. Reaming of human tibial pseudoarthrosis seems to be beneficial (Alho et al. 1993), but the effect of reaming of fresh fractures is more uncertain (Mayr et al. 1995), which may be because the signal substances are already present. Our study indicates that temporary ischemia, as caused by intramedullary reaming, could improve callus formation. Another interesting task would be to identify the mediators released by the ischemic insult. These mediators may be released by ischemic bone or surrounding soft tissue (muscle), granulocytes, monocytes, macrophages or other multipotent cells. Our series indicates that osteoprogenitor cells in the periosteum may be activated by transient ischemia without necrosis of the bone or periosteum. The relative importance of cells from extraosseous tissues, bone and periosteum in the healing of diaphyseal fractures has not yet been resolved, but it has been claimed that callus is mainly derived from a remnant of periost or from the edges of bone with intact periosteum (Oni and Gregg 1991). According to our findings, these parts may start to proliferate in response to transient ischemia.

In conclusion, our hypothesis suggesting increasing periosteal hypertrophy and hyperplasia with increasing ischemic insult was verified. This response occurred only when the ischemia was severe enough to cause extensive muscle necrosis, but was aborted if it was severe enough to cause necrosis of the bone.

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