

Periosteal response to transient ischemia

Histological studies on the rat tibia

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Complete arrest of blood flow was induced for 4.5 hours in the left hindlimb of Wistar rats by a tourniquet applied proximally to the thigh. Histologically the periost of the tibial bone after 3 days showed marked hypertrophy and hyperplasia of the peri-

osteal cells with an osteogen differentiation. This response is similar to the initial formation of external callus during fracture healing. Transient ischemia may be an important factor in initiating fracture healing.

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Bending and compression initiate bone formation on the periosteal surface (McKibbin 1978, Lanyon and Rubin 1984, Göransson et al. 1992, Raab et al. 1993, Torrance et al. 1994). The mechanism of this response has been attributed to local strain in the bone, but it has also been suggested that the loading disrupts or compresses blood vessels leading to local tissue hypoxia (Raab et al. 1993). A venous tourniquet increases bone formation in fractures (Kruse and Kelly 1974). The ischemic effect of venous hypertension is, however, uncertain.

We examined the periosteal response in rat tibia after transient hindlimb ischemia induced in the proximal thigh by a modified tourniquet model.

Animals and methods

Surgical techniques

Male Wistar/Han/Mol SPF rats, weighing 250–300 g, were anesthetized with a combination of fentanyl 0.05 mg/mL, fluanisone 2.5 mg/mL and midazolam 1.25 mg/mL. Initially, 2.3 mg/kg were given subcutaneously, followed by 1.7 mL/kg/h during the period of ischemia and early reperfusion.

We combined open clamping of the femoral artery with application of tourniquets around the proximal thigh to occlude all collateral circulation, without compression of main vessels and nerves (Skjeldal et

al. 1991).

The femoral veins, arteries and nerves were dissected free distal to the left inguinal ligament, and the femoral arteries occluded with microvascular clips. The sciatic nerves were dissected free through separate incisions proximally on the thigh. 2 silicone tubings 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 animals were placed in an infant incubator (Air-Shields Europe, Shannon, Ireland) and kept at 27°C during ischemia and early reperfusion. The tourniquets and clips were released after 4.5 h of ischemia, and 0.5% lidocaine chloride was applied locally to prevent spasms in the vessels. All animals, including the controls (see below), were given 5 mL of 0.9% NaCl intraperitoneally (i.p.) at the end of the ischemic period to prevent dehydration. 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.

Histological investigations

72 hours after the ischemic insult the animals were killed with fentanyl/fluanisone/midazolam, and the left leg was exarticulated in the hip. The legs were fixed in 4% buffered formaldehyde and decalcified in 30% hydrochloric acid for 24 hours. Tissue blocks from the geometric middle third of the lower leg were

embedded in paraffin, and 4-micron thick cross-sections were stained with hematoxylin and eosin. The section plane was perpendicular to the long axis of the tibia (Figure 1).

The periost was defined as a 2-layer membrane: an outer fibrous and an inner osteogenic layer as described by Ham and Harris (1971). The number of lining cells in these 2 layers of periost was counted at 4 different locations with an eye-piece graticule, and the mean value was calculated (Figure 1). The countings were repeated 4 times. The coefficient of variation for the measurements varied from 1.4 to 4.3 (SD/mean X 100).

The periosteal areas were measured with computerized morphometry (Kontron Mini Mop Microprocessor, Kontron Bioanalyse, Munich, FRG) and calculated in per cent of the total cross-sectional area of tibia (periost, bone and marrow tissue).

Experimental groups

12 rats were randomized into 2 groups of 6 animals each.

The ischemic group was subjected to 4.5 hours of ischemia at 27 °C. The control group was left untouched until they were given an anesthetic and killed.

All animals were in good condition until they were killed 3 days after the operation.

Statistics

The bone area and area of periost and the number of cells in the periost are given as medians and standard deviations (SD). The areas in the two groups were compared using the Mann-Whitney U test, and p <0.05 was considered significant.

Results

The ischemic animals exhibited a hyperemic response in the skin after restoration of the flow, and a pronounced subcutaneous edema later indicated reperfusion. The cross-sections showed a sharp demarcation between the periost and the bone tissue in both ischemic and control animals.

1 animal in the control group was discarded because of a technical error in the preparation.

In the control group, the tibial bone was covered by a fibrous periost consisting of 2–3 cell layers (Table 1). The cells were spindle-like and indistinguishable from normal fibroblasts. No osteogenic layer was visible (Figure 2), and the bone and bone marrow tissues were normal. The marrow cavity was lined with a distinct cell layer of cuboid endosteal

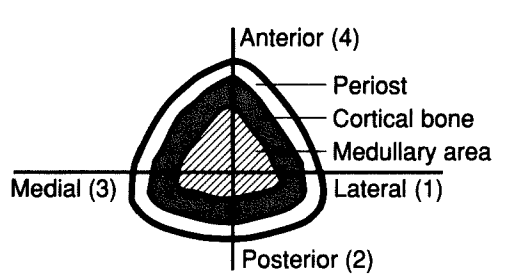


Figure 1. Tibial cross-section and locations for counting (1–4) of periosteal cells.

Table 1. Number of cells in the periost counted at four different locations in rat tibias in control rats and in rats subjected to 4.5 h hindlimb ischemia

Group (animal no.)	Fibrous layer Location					Osteogenic layer Location				
	1	2	3	4	Median	1	2	3	3	Median
<i>Control</i>										
1	2	3	2	2	2	0	0	0	0	0
2	3	3	4	2	3	0	0	0	0	0
3	4	2	2	2	2	0	0	0	0	0
4	2	2	2	2	2	0	0	0	0	0
5	4	3	3	3	3	0	0	0	0	0
Median					2					0
<i>Ischemic</i>										
1	2	2	2	2	2	3	11	9	6	7.5
2	2	3	5	4	3.5	25	18	21	22	22
3	4	5	2	3	3.5	24	8	20	15	18
4	5	4	3	2	3.5	16	10	7	7	8.5
5	4	2	2	2	2	10	5	4	5	5
6	3	4	3	2	3	15	10	13	20	14
Median					3.5					11.3*

*P 0.004, ischemic versus control

cells, and no proliferation or hyperplasia of the endosteum was found.

The animals subjected to ischemia showed marked histopathological changes in the periosteum. The osteogenic cell layer dominated with both hyperplasia and hypertrophy of the cells (Figure 2). These hypertrophic cells showed typical changes of increased activity, with enlargement of the nuclei and prominent nucleoli (Figure 3). The cells in the part close to the bone showed differentiation into osteoblasts, surrounded by new immature bone (Figure 4).

The fibrous layer was 3 (mean)-cells thick and lined the bone as a membrane. The inner osteogenic layer, however, varied from 6 to 21 cells in thickness, with a mean value of 13 cells (Table 1).

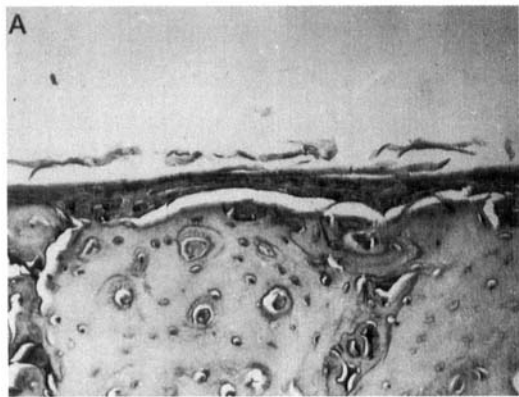
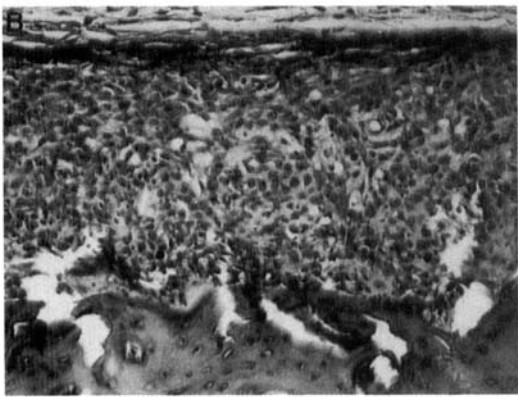


Figure 2. A. Cross-section of the tibial bone from a rat in the non-ischemic control group.



B. The tibial bone in a rat subjected to 4.5 hours of hindlimb ischemia and 72 hours of reperfusion. Note the periosteal hyperplasia (HE, $\times 200$).

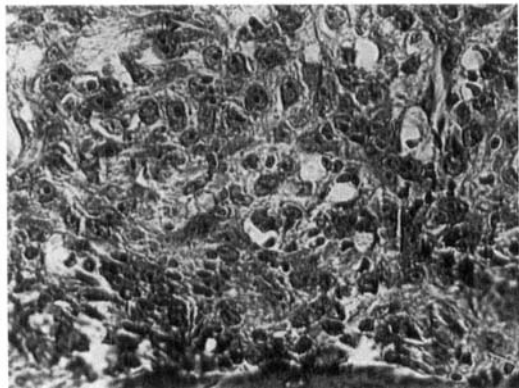


Figure 3. Photomicrograph of the periosteal response after 4.5 hours' ischemia and 72 hours' survival with hypertrophic cells containing large nuclei and prominent nucleoli (HE, $\times 500$).

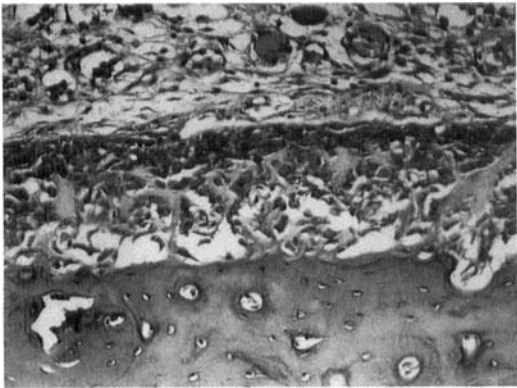


Figure 4. Photomicrograph demonstrating the innermost layer of the reactive periosteum. Immature bone trabeculae are cemented to the old bone (HE, $\times 200$).

Table 2. Bone areas and areas of periosteum in cross-sections of the tibia in the control group and after 4.5 h transient ischemia

Group (animal no.)	Bone area (mm ²)	Periosteal area (mm ²)	Ratio periosteum/bone (%)
<i>Control</i>			
1	4.91	0.33	7
2	4.88	0.14	3
3	5.56	0.22	4
4	7.81	0.46	6
5	6.45	0.32	5
Median	5.6	0.3	5
<i>Ischemic</i>			
1	4.07	0.46	11
2	9.18	1.99	22
3	9.59	1.48	15
4	6.22	0.72	12
5	4.61	0.7	15
6	6.05	1.3	21
Median	6.2	1.3 ^a	15.4 ^b

^a P 0.008, ^b P 0.006 ischemic versus control

All the specimens in the operated animals showed morphologic normal lamellar bone with osteocytes in the lacunae. No significant endosteal activation was found and the bone marrow showed necrotic hemopoietic tissue. The surrounding muscles also showed extensive necrosis.

In the control group, the mean periosteal area was 5 (3-7) percent of the total cross-sectional area of the tibia. While the periosteum in the control tibias represented a small bone border, it had increased significantly in the rats subjected to ischemia. The periosteum in this group contributed to 16 (11-22) percent of the total area (Table 2).

The mean cell number in the osteogenic layer correlated closely with the periosteal area in the ischemic group by (r 0.97, p 0.001).

Discussion

We found a marked periosteal response to transient hindlimb ischemia in rats. While other tissues in the leg underwent partial necrosis, the periost showed aggressive proliferation.

The histopathological changes consisted of hypertrophy and hyperplasia of the cells, with differentiation into osteoblasts in the deeper part of the periosteum and an unchanged outer fibrous layer. This distinct cellular response and the bone differentiation found 3 days after the ischemic period are similar to the external callus in fracture healing (Kernek and Wray 1973, Ham and Harris 1971, McKibbin 1978). Animal studies have shown that activation of cells in the periost appear about 24 hours after a fracture (Tonna and Cronkite 1961, Aho 1966), and that periosteal bone formation takes place within 48 hours (Aho 1966, Kernek and Wray 1973).

Chondrogen transformation has been noted in the callus a few days after bone trauma (Hulth and Olerud 1964, Aho 1966, Kernek and Wray 1973, McKibbin 1978, Göranson et al. 1992). We found no cartilage in the hyperplastic periost, but the animals survived only for 3 days.

It is not resolved which extraosseous cells are most important in fracture healing (Hulth 1990). Several studies have suggested that callus forms mainly from pluripotent cells (Oni and Gregg 1990, Rand and Bergquist 1992), while others point out the importance of the periosteum (Ham and Harris 1971, Oni and Gregg 1990).

According to McKibbin (1978), the initial periosteal responses of injured bones are identical in fractures, in amputation stumps and bones that are merely injured without producing a complete fracture. In our study there was no local injury to the lower leg. Trauma by itself is therefore not a requisite for periosteal activation.

Our findings support the view that transient ischemia initiates bone formation, as suggested by others (Hutchinson and Burdeaux 1954, Raab et al. 1993). Furthermore, our study shows histological similarities between the ischemic periosteal response and the bone formed after mechanically applied forces (Aho 1966, Ham and Harris 1971, Kernek and Wray 1973, McKibbin 1978).

In our study the ischemia was transient, and the leg was reperfused for 3 days before the animals were killed. Several studies have related the postischemic damage to the adequacy of the reperfusion (Engler et al. 1986, Gidlöf and Lewis 1990). The reestablishment of blood flow is postulated to aggravate the tissue damage. We did not measure the blood flow to the

tibial bone after the ischemic period, but neither the bone tissue nor the periosteum was damaged or necrotic. The periosteum rather showed increased general activity. Furthermore, the necrotic bone marrow was surrounded by an almost inactive endosteum.

Our model gives complete arrest of blood flow to the leg and, consequently, tissue hypoxia (Skjeldal et al. 1991). The transient hypoxia may be an important factor in initiating activation of the periosteum. Raab et al. (1993) found a periosteal response after static, but not cyclic compression to animal limbs, and supposed it to be due to local tissue hypoxia caused by prolonged tissue compression. Girgis and Pritchard (1958) and Ham and Harris (1971) believed that inadequate blood supply at the fracture site leads to the formation of cartilage. Shaw and Basset (1967) have also demonstrated the importance of the amount of available oxygen in cellular behavior of bone and cartilage cells. Brighton and Krebs (1972) showed that in healing fractures both cartilage and bone are formed in areas of low oxygen tension.

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