THE EFFECTS OF EXTRAPERIOSTEAL INJECTIONS OF BLOOD COMPONENTS ON PERIOSTEAL CELL PROLIFERATION

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ABSTRACT

Following extraperiosteal injections of saline, serum, or whole blood, the synthesis of DNA in the cells of the osteogenic layer of the femora of mice was stimulated to approximately two-thirds of the level obtained by fracture of the femora. Irrespective of the material injected, the proliferative response of the cells in the periosteum was similar. These studies have shown that neither bone fracture nor direct disruption of the periosteum is necessary to induce periosteal cell proliferation since a single extraperiosteal injection of physiological saline induces DNA synthesis.

The periosteum prenatally has a high level of proliferative activity that is intensified during the peak of bone growth and development and then becomes considerably diminished within a short period of time. However, the ability of the periosteum to undergo renewed proliferation in response to trauma is not diminished with age as abruptly as the respiratory enzyme activity, the mitochondrial population, or the cell proliferative activity required for normal growth (1–7). The femoral periosteum of the mouse possesses a high degree of activity during the relatively short period of intense growth following birth, but retains a lifelong reparative potential.

The objectives of the present investigation were to determine which, if any, of the components of the fracture exudate were responsible for the stimulation of periosteal proliferation after fracture, or whether stimulation was purely due to sequelae of local trauma (edema, local hypoxia, exudation, nervous stimuli from pain, etc.).

The present study resulted from earlier observations in which periosteal proliferation after trauma appeared not only at the fracture site but along the entire femoral shaft (6, 7). Since the exudate resulting from trauma extended beyond the fracture site, it was hypothesized that some component of the exudate may either directly or indirectly stimulate periosteal proliferation.

MATERIALS AND METHODS

Experiment I

Thirty-six 5- to 6-week-old female mice of the Brookhaven National Laboratory inbred strain of Swiss albino were used. Mice were randomized in groups of 6. An additional group containing 12 mice was used to supply fresh pooled whole blood and serum. Groups consisted of A controls, B saline-injected, C whole blood-injected, and D serum-injected. The left femora of mice in all groups were fractured at mid-shaft by digital pressure, while the right femora of mice in groups B, C, and D were injected at mid-shaft with 0.25 ml of sterile physiological saline, pooled fresh mouse whole blood containing a small amount of (1.5 per cent) EDTA in 0.7 per cent saline, or freshly prepared mouse serum, respectively. Injections were made with a 24-gauge needle inserted into the soft tissues above the periosteum, near the mid-shaft region of the femur, endeavoring not to damage the periosteum directly. Twenty-nine hours later all the mice were given a

subcutaneous injection of tritiated thymidine (H^3TDR)[1] (0.5 μc per gram of body weight). One hour later the mice were killed with ether. Whole femora were removed intact with the surrounding soft tissues and fixed in cold (4°C) buffered formalin (pH 7) for 24 hours, followed by 24 hours of washing in running tap water. Bones were decalcified in 10 per cent Versene, dehydrated, and imbedded in paraffin. Sections were then cut at 5 microns. Autoradiographs were prepared by dipping tissue sections in Kodak NTB_3 liquid emulsion and exposing the preparations in the dark, in a cold, dry atmosphere for 16 days. After exposure, the emulsions were developed for 2 minutes in D-19 developer, briefly rinsed in water, fixed for 2 minutes in hypo solution, and washed in running tap water for 30 minutes. The slides were finally stained with hematoxylin.

Experiment II

In order to obtain whole blood with very few white blood cells, 12 mice were exposed to 876 rads of whole body radiation, at 20.4 rads per minute, from a Co^{60} source. This dose produced 100 per cent mortality within 17 days in our strain of mice. Whole blood of 6 mice was pooled 30 hours after irradiation, while the whole blood of the remaining 6 mice was pooled 6 days after irradiation.

Eighteen additional mice were divided into three groups. Group E represented a repeat of Group A in Experiment I, since Experiment II was performed at a different time period. Group F consisted of non-irradiated mice. These animals were injected as in Experiment I, but with 0.25 ml of fresh pooled whole blood removed from irradiated mice, 30 hours post-irradiation. The left femora were immediately fractured. Twenty-nine hours later the mice were given H^3TDR and killed 1 hour later. Group G mice were similar to Group F and were treated in a similar manner except that they were given 0.25 ml of fresh pooled whole blood removed from irradiated mice, 6 days after irradiation. The left femora were also fractured. H^3TDR was given 29 hours later, and at the 30th hour the mice were killed. Blood counts were made with a Coulter Counter from the irradiated Group and Group E mice prior to fracture, and 30 hours after fracture. The mice of Experiment II were similar in age to those of Experiment I. Tissues taken from Groups E, F, and G were also treated as in Experiment I.

In both experiments, the number of tritium-labeled cells (N^*) and the total cell population (N) of the osteogenic layer of the femoral periosteum were counted in autoradiographs to obtain the "Labeling Index" ($L_i = N^*/N$), which, when multiplied by 100, gave the values referred to as per cent of cells labeled. A period of 30 hours after fracture was chosen for the observation point, since it was shown previously in young mice (6) that a maximum proliferative response of periosteal cells to trauma was reached at about this time.

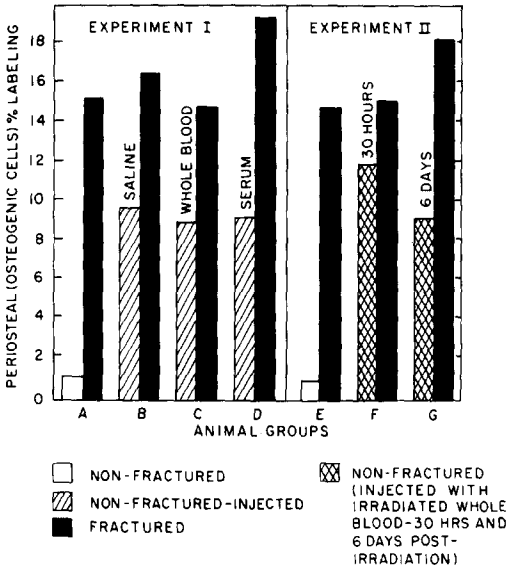

FIGURE 1 The bar graph represents the per cent labeling index of the osteogenic layer of the periosteum of different experimental groups. Each group is represented by two columns; one for the non-traumatized femora, the other for the fractured femora. Note, in Experiment I, the constancy in response to the extra-periosteal injection of saline, whole blood, or serum.

RESULTS

Experiment I

AUTORADIOGRAPHY: In the non-fractured controls (Group A), 1 per cent of the periosteal osteogenic cells was seen labeled; the frequency was higher in the preosteoblastic segment of the population (Fig. 2). Occasionally, labeled cells of the fibrous layer were also observed. Labeled cells were more frequently noted at the perichondrial regions of the femur. Extensive labeling of periosteal cells (average 16.4 per cent) was noted in the fractured femora of all groups 30 hours after trauma (Fig. 3). Labeled cells were distributed along the shaft in the osteogenic layer of the periosteum. A typical extraperiosteal inflammatory exudate containing numerous large labeled mesen-

[1] Tritiated thymidine (Specific Activity 1.9 curies/mM in sterile aqueous solution) was obtained from Schwarz Bioresearch Inc., Orangeburg, New York.

chymal cells was also observed. Periosteal labeling appeared to be paralleled by the presence of the inflammatory exudate.

The autoradiographs of injected femora resembled those of the fractured femora except that the over-all periosteal response was less intense (average 9.2 per cent). No variations were noted

counts made before and after femoral fracture of the mice of control Group E (average count 9,415/mm³) also showed no significant difference. However, the leucocyte count made 30 hours after irradiation had fallen to 2,883/mm³ (see Table II). A paucity of lymphocytes was seen in blood smears following irradiation.

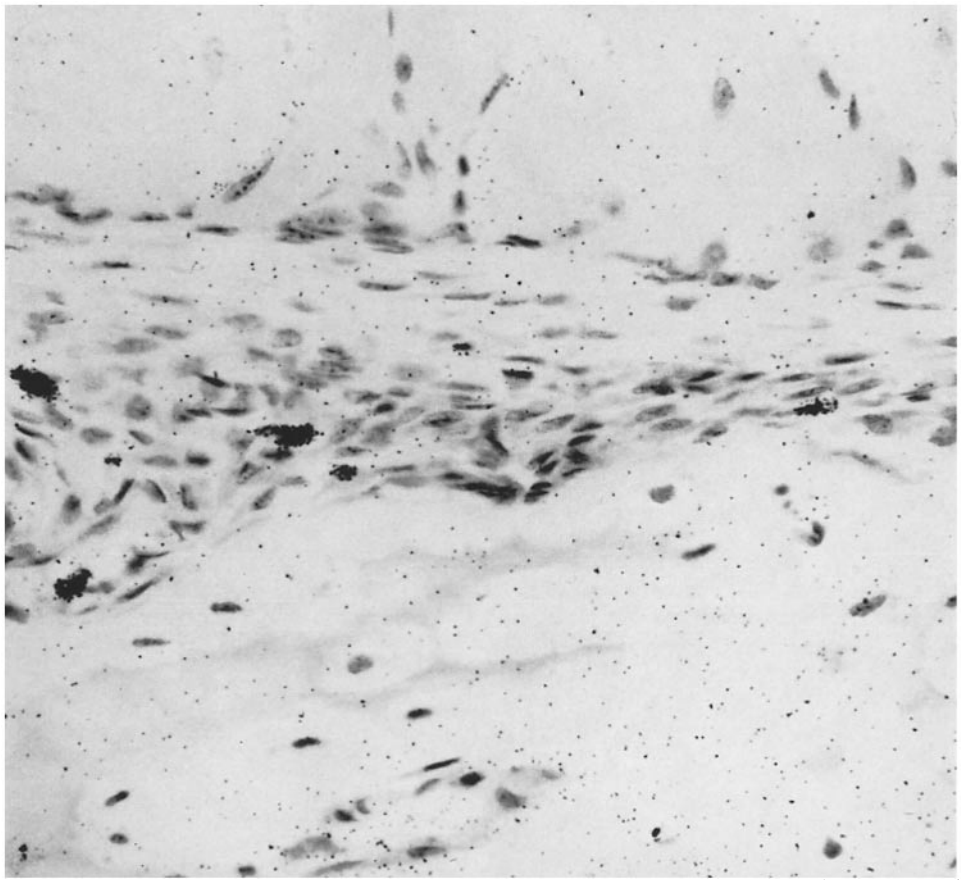

FIGURE 2 An autoradiograph of the periosteum of a non-fractured control femur. Labeling is generally limited to a few osteogenic cells. The autoradiographs have been overexposed in order to increase the density of labeling in cells. Harris hematoxylin-stained. × 300.

in the labeling of different cell types or in the intensity of labeling of femora injected with saline (Fig. 4), fresh pooled whole blood, or serum (Table I and Fig. 1).

Experiment II

BLOOD COUNTS: No significant differences were obtained in red cell counts between groups (average count 7.23 × 10^6/mm³). White cell

AUTORADIOGRAPHY: No significant difference in the per cent labeling of the periosteum was seen between control Group E of Experiment II and control Group A of Experiment I. In the non-fractured femora of Group E, 14.7 per cent of the periosteal cells were labeled.

In the autoradiographs of Groups F and G which were injected with irradiated blood, a high level of periosteal labeling was noted. The levels

were similar to those observed in the injected mice of Experiment I. The periosteum of the non-fractured femora of Group F was 11.9 per cent labeled. In the injected femora of Group G, 9.1 per cent of the periosteal cells were labeled, whereas 15.1 and 18.3 per cent labeling was observed in the periosteum of the fractured femora of Groups F and G, respectively. Extraperiosteal labeling was similar to that of Groups B, C, and D. The labeling data are tabulated in Table III and plotted in Fig. 1.

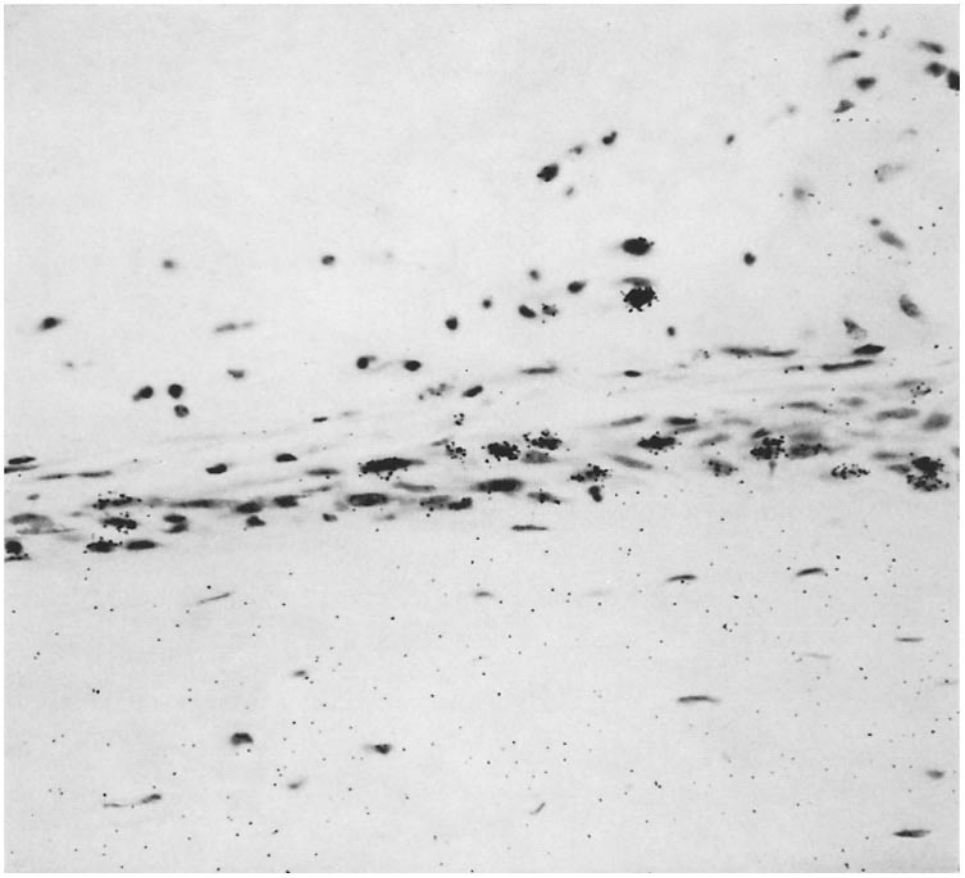

FIGURE 3 An autoradiograph of the periosteum of the fractured control femur, 30 hours after trauma. Labeling of the osteogenic layer is extensive. Osteoblasts as well as pre-osteoblasts are seen labeled. The autoradiographs have been overexposed in order to increase the density of labeling in cells. Harris hematoxylin-stained. × 300.

DISCUSSION

These experiments were undertaken to ascertain the influence of three factors which may be responsible for periosteal cell proliferation following fracture, namely: (1) the release of a component contained within the blood which blocks the action of an endogenous inhibitor of proliferation within the periosteum, (2) the introduction of a component from white blood cells, platelets, or serum proteins which stimulates DNA synthesis directly, or (3) the formation of a positive stimulus resulting from local hypoxia or local activation of enzymes by serum or blood cell activators.

The present study has demonstrated clearly that stimulation of periosteal proliferation is not dependent upon bony fracture or disruption of the periosteum, since an extensive fracture-like response was produced following extraperiosteal injections of saline and several blood components.

Apparently, the magnitude of the proliferative response depended upon the extent of adjoining extraperiosteal disturbance caused by the injection. Since saline alone produced a response similar to that elicited by injections of whole blood or serum, one must search for a common factor which is associated with fracture.

Although this report is concerned with the

TABLE I

Per cent Labeling of Mouse Femoral Periosteum 1 Hour After Tritiated Thymidine Administration and 30 Hours Following Extraperiosteal Injections of 0.25 ml of Blood

Animal groups & treatment	Right femur			Left femur	
	Non-fractured (Normal)	Injected (Non-fractured)	No. cells counted	Fractured	No. cells counted
				30 hrs.	
A—Controls	1.0	—	11,500	15.2	9,532
B—Saline	—	9.6	4,115	16.5	2,560
C—Whole Blood	—	8.8	3,200	14.7	1,760
D—Serum	—	9.1	2,275	19.3	2,019

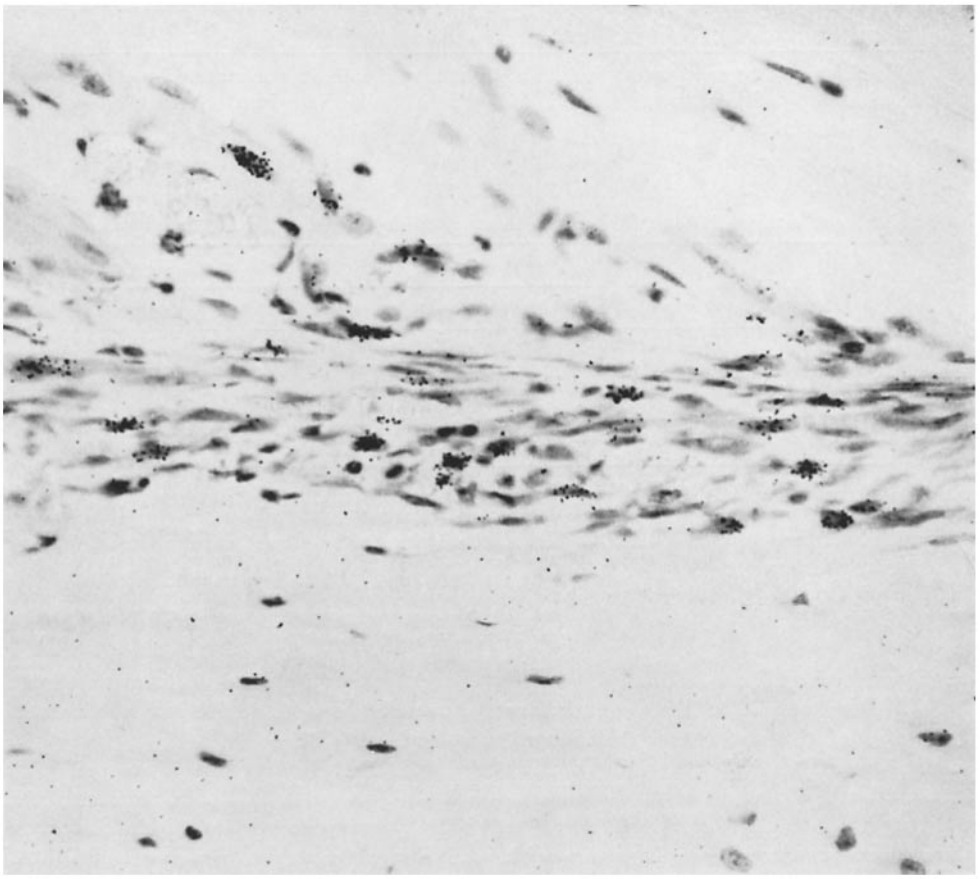

FIGURE 4 An autoradiograph of the periosteum of a non-fractured femur which 30 hours previously had been injected extraperiosteally with 0.25 ml of sterile saline. Note that cell proliferation has also been stimulated extraperiosteally. Harris hematoxylin-stained. × 300.

stimulation of DNA synthesis, a brief review of certain findings which deal with osteogenesis and bone induction may be pertinent. There is an extensive literature on postfetal osteogenesis (8–10) which contributes little to an understanding of cell proliferation in the periosteum following fracture. From studies in which bone, cartilage, fracture callus, and marrow were transplanted into the anterior chamber of the eye, it was concluded that vital skeletal tissues are required to induce osteogenesis (11–14). However, other workers (15) have used devitalized skeletal tissue and have observed that osteogenesis was induced, thus indicating that vital skeletal tissues are not required. Still others have believed that osseous tissue contains an inductor of new bone formation and have tried to induce bone formation with osseous extracts, with conflicting results (8, 16–24). For simplicity, it is better to assume that there exists a single common factor underlying osteogenesis. For diverse reasons, however, it is unlikely that this factor resides solely within bone. If it does, it must be transported elsewhere either by blood cells or by plasma, since bone formation can be induced by bladder wall (25–28), rabbit muscle (29), and it also occurs in old tuberculous lesions. In these cases, the formation occurs in tissues which do not originate in bone. However, the possibility remains that primitive mesenchymal cells are involved and that either local factors induce these cells to proliferate into osteogenic tissue or osteogenic inductors are somehow attracted and transported into the area of the induction.

Along the previous line of reasoning, studies with diffusion chambers seem relevant, since new bone growth was induced within chambers containing mouse calvaria which were implanted into subcutaneous tissue without external bone formation (30). In this case, it appears as though osteogenesis was induced from host tissue or by alteration of the milieu of the cells within the

TABLE II

*Blood Counts of Irradiated and Non-Irradiated Mice**

Animal groups & treatment	WBC ± AD	RBC ± AD
	Count/mm³	Count × 10^6/mm³
E—Controls (before fracture)	10,083 ± 1.068	7.36 ± 0.09
E—Controls (30 hrs. postfracture)	8,747 ± 330	7.51 ± 0.27
Irradiated mice (30 hrs. postirrad.)	2,883 ± 643	7.21 ± 0.26
Irradiated mice (6 days postirrad.)	1,345 ± 168	6.82 ± 0.51

* Each value represents an average of 18 counts (6 mice, 3 per mouse).
Irradiation: Co^{60} source acute whole body dose of 876.4 rads given at a dose rate of 20.4 rads/min.

TABLE III

Per cent Labeling of Mouse Periosteum 1 Hour after Tritiated Thymidine Administration

Animal groups & treatments	Right femur		Left femur	
	Non-fractured	No. cells counted	Fractured	No. cells counted
			30 hrs.	
E—Controls	0.8	4,480	14.7	2,800
F—Non-irradiated (30 hrs.)	11.9*	3,889	15.1	3,200
G—Non-irradiated (6 days)	9.1†	3,740	18.3	3,000

* Right femora were injected with pooled whole blood of irradiated mice killed 30 hrs. postirradiation.
† Right femora were injected with pooled whole blood of irradiated mice killed 6 days postirradiation.
Irradiation: Co^{60} source—acute whole body dose of 876.4 rads given at a dose rate of 20.4 rads/min.

chamber (pH, ionic distribution, metabolites, etc.), and it must be remembered that diffusion distances from capillaries are greatly increased and oxygen diffusion is very poor (31). *In vitro* studies (32) with very thin filters have shown that induction with various types of tissues can be produced. It was believed that induction involved colitis, for example). Correction of the basic disease may result in the subsidence of the HPO as well as the periostitis in those cases with increase in venous pressure or arterial undersaturation. There appears to be some logical connection between circulatory disturbances and stimulation of periosteal cell proliferation. However, in the

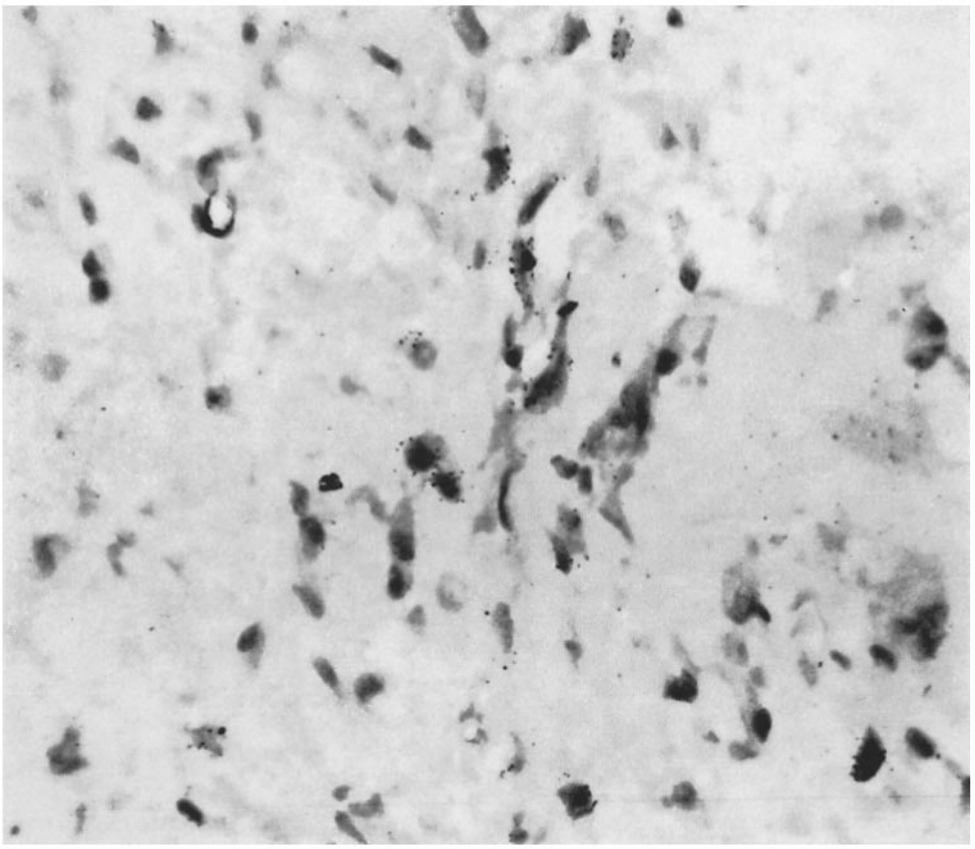

FIGURE 5 An autoradiograph of the femoral periosteum of an adult dog (boxer), with hypertrophic pulmonary osteoarthropathy, 1 hour after injection with 0.1 μc H^3TDR/gm body weight, showing extensive labeling of cells. Harris hematoxylin-stained. × 500.

interaction between intercellular proteins and polysaccharides passing through the filters (33).

The pathogenesis of hypertrophic pulmonary osteoarthropathy (HPO) (34) is believed to be related to factors which induce periosteal proliferation after trauma. Many cases of HPO follow chronic pulmonary disease which induces hypoxia as a result of venous stasis or arterial undersaturation with oxygen. However, some cases of HPO result from chronic liver or intestinal disease (amebic abscesses of liver or chronic ulcerative case of hepatic or gastrointestinal disease, the cause and effect relationship is obscure and one invokes the possible existence of a toxin as the cause of the histologic appearance of HPO. Evidence from an experiment now in progress, in which an adult dog (boxer) with HPO was injected with 0.1 μc H^3TDR/gm of body weight (Fig. 5), shows that the periosteum was in a state of active proliferation. In both HPO-induced periostitis and stimulation of periosteal cell proliferation following extraperiosteal injections of

saline, evidence of bone and periosteal trauma is absent, as far as can be assessed.

Carnot and Deflandre (35) proposed the theory that hypoxia elicited the formation of humoral factors (erythropoietins) which stimulated erythropoiesis. The theory was confirmed by Reissmann (36) using parabiotic rats in which one partner was exposed to a low O_2 environment. Both animals revealed stimulated erythropoiesis. If a similar mechanism exists in stimulating periosteal cell proliferation, it would not explain why the response occurs only at the traumatized bone nor explain osteoarthropathy arising from conditions which exhibit apparently normal venous pressure and normal arterial oxygen saturation. On the other hand, if, following trauma, local hypoxia produced a tissue factor which can stimulate periosteal cell proliferation in an altered environment, then the observed local periosteal behavior can be explained.

We are, therefore, extending these studies in order to determine the effects of hypoxia on periosteal cell proliferation and postfetal osteogenesis based on the working hypothesis, namely, that extra- or intraperiosteal capillary damage, following trauma, produces local hypoxia which results in the formation of a local tissue factor capable of stimulating periosteal cell proliferation in the trauma-altered environment.

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BIBLIOGRAPHY

1. TONNA, E. A., Histologic and histochemical studies on the periosteum of male and female rats at different ages, *J. Gerontol.*, 1958, **13**, 14.
2. TONNA, E. A., Respiration and the skeletal system, *Bull. Hosp. Spec. Surg.*, 1959, **2**, 50.
3. TONNA, E. A., Post-traumatic variations in phosphatase and respiratory enzyme activities of the periosteum of aging rats, *J. Gerontol.*, 1959, **14**, 159.
4. TONNA, E. A., The cellular complement of the skeletal system studied autoradiographically with tritiated thymidine (H^3TDR) during growth and aging, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 813.
5. TONNA, E. A., and PILLSBURY, N., Mitochondrial changes associated with aging of periosteal osteoblasts, *Anat. Rec.*, 1959, **134**, 739.
6. TONNA, E. A., and CRONKITE, E. P., Cellular response to fracture studied with tritiated thymidine, *J. Bone and Joint Surg.*, 1961, **43A**, 135.
7. TONNA, E. A., and CRONKITE, E. P., Changes in skeletal cell proliferative response to trauma concomitant with aging, *J. Bone and Joint Surg.*, 1962, **44A**, 1557.
8. BERTELSEN, A., Experimental investigations into post-fetal osteogenesis, *Acta Orthop. Scand.*, 1944, **15**, 139.
9. MCLEAN, F. C., and URIST, M. R., *in* Bone, an Introduction to the Physiology of Skeletal Tissues, 2nd edition, University of Chicago Press, 1961.
10. MAKIN, M., Ostoegenesis induced by vesical mucosal transplant in the guinea pig, *J. Bone and Joint Surg.*, 1962, **44B**, 165.
11. PFEIFFER, C. A., Development of bone from transplanted marrow in mice, *Anat. Rec.*, 1948, **102**, 225.
12. RAY, R. D., DEGGE, J., GLOYD, P., and MOONEY, G., Bone regeneration. An experimental study of bone-grafting material, *J. Bone and Joint Surg.*, 1962, **34A**, 638.
13. URIST, M. R., and MCLEAN, F. C., Osteogenetic potency and osteogenetic inductor substances of periosteum, bone marrow, bone grafts, fracture callus, and hyaline cartilage transferred to the anterior chamber of the eye, Conference on Metabolic Interrelations, 3rd Meeting, New York, Josiah Macy Jr. Foundation, 1951, 55.
14. URIST, M. R., and MCLEAN, F. C., Osteogenetic potency and new-bone formation by induction in transplants to the anterior chamber of the eye, *J. Bone and Joint Surg.*, 1952, **34A**, 443.
15. BRIDGES, J. B., and PRITCHARD, J. J., Bone and cartilage induction in the rabbit, *J. Anat.*, 1958, **92**, 28.
16. HEDRI, R., Ein neues prinzip der osteosynthese, *Arch. klin. Chir.*, 1931, **167**, 145.
17. LEVANDER, G., An experimental study of the role of the bone marrow in bone regeneration, *Acta Chir. Scand.*, 1940, **83**, 545.
18. LEVANDER, G., and WILLISTAEDT, H., Alcohol—soluble osteogenic substance from bone marrow, *Nature*, 1946, **157**, 587.
19. WILLSTAEDT, H., LEVANDER, G., and HULT, L.,

Studies on osteogenesis, *Acta Orthop. Scand.*, 1950. **19,** 419.

20. LACROIX, P. The Organization of Bones, Philadelphia, Blakiston, 1951.
21. LINDAHL, O., and ORELL, S., Experiments with bone extract, *Acta Chir. Scand.*, 1951, **101,** 136.
22. HARTLEY, J., and TANZ, S. S., Experimental osteogenesis in rabbit muscle, *Arch. Surg.*, 1951, **63,** 845.
23. MOSS, M. L., Experimental alteration of sutural area morphology, *Anat Rec.*, 1957, **127,** 569.
24. MOSS, M. L., Extraction of an osteogenic inductor factor from bone, *Science*, 1958. **127,** 755.
25. HUGGINS, C. B., The formation of bone under the influence of epithelium of the urinary bladder, *Arch. Surg.*, 1931, **22,** 377.
26. HUGGINS, C. B., The phosphatase activity of transplants of the epithelium of the urinary bladder to the abdominal wall producing heterotopic ossification, *Biochem. J.*, 1931, **25,** 778.
27. HUGGINS, C. B., and SOMMET, J. F., Function of the gallbladder epithelium as an osteogenic stimulus and the physiological differentiation of connective tissue, *J. Exp. Med.*, 1933, **48,** 393.
28. BELL, J. W., and KNUDTSON, K. P., Soft tissue osteogenesis with gall-bladder mucosal autografts, *Lab. Invest.*, 1961, **10,** 397.
29. HEINEN, J. H., JR., DABBS, G. H., and MASON, H. A., The experimental production of ectopic cartilage and bone in the muscles of rabbits, *J. Bone and Joint Surg.*, 1949, **31A,** 765.
30. GOLDHABER, P., Preliminary observations on bone isografts within diffusion chambers, *Proc. Soc. Exp. Biol. and Med.*, 1958, **98,** 53.
31. WARBURG, O., Versuche an überlebendem Carcinomgewebe, *Biochem. Z.*, 1923, **142,** 317.
32. GROBSTEIN, C., *in* Aspects of Synthesis and Order in Growth, (D. Rudnick, editor), Princeton University Press, 1954, 233.
33. GROBSTEIN, C., *in* Discussion of the paper by Peter J. Gaillary, Morphogenesis in Animal Tissue Cultures, *J. Nat. Cancer Inst.*, 1957. **19,** 600.
34. BAUER, W., *in* Textbook of Medicine, (R. L. Cecil and R. F. Loeb, editors), Philadelphia, Saunders Co., 1951, 1358.
35. CARNOT, P., and DEFLANDRE, C., Sur l'activité hémopoiétique du sérum au cours de la regenération du sang, *Compt. rend. Acad. sc.*, 1906, **143,** 384.
36. REISSMANN, K. R., Studies on the mechanism of erythropoietic stimulation in parabiotic rats during hypoxia, *Blood*, 1950, **5,** 372.