Histological and Alkaline-Phosphatase Changes in Autogenous Transplants of Tibial Grafts in Dogs Treated with Cortisone*

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A better understanding of the effects of corticosteroids on the mineralization and repair of bone is necessary with the increasing use of these substances in patients with orthopaedic problems. The profound effects of cortisone on various metabolic activities were shown by Hench and associates and Layton. Some investigators have believed that there is a delay in bone repair after treatment with compound E. Key, Odell, and Taylor, however, were not able to demonstrate this delay after cortisone. Dosage and animal resistance appear to be factors in this situation.

Duthie and Barker used rats in their studies. They administered one milligram of cortisone per fifty grams of body weight. Key, Odell, and Taylor failed to demonstrate any alterations in the repair of experimental fractures in rats, using cortisone in doses of about one milligram per fifty grams of body weight in one group, and about one milligram per 100 grams in another group. However, they did observe a persistence of hemorrhage at the fracture site in cortisone-treated rats. Blunt and associates, on the other hand, found rabbits to be very sensitive to approximately eight milligrams per kilogram of body weight of cortisone daily, whereas Curtiss did not find transplants in dogs to be particularly altered by one to two milligrams of cortisone per kilogram of body weight.

The treatment of rabbits with cortisone revealed an increased resorption of bone, whereas halting the treatment was followed by a rapid development of bone. Storey also noted that each new layer of bone was demarcated by a "reversal" line as a consequence of the repeated waves of resorption and deposition of bone after intermittent treatment with cortisone.

Duthie and Barker, studying fracture healing in rats with autoradiograms, indicated that cortisone affects the formation of periosteal cartilaginous blastema and thus the process of endochondral ossification, with liberation of increased amounts of chondroitin sulphate which was calcified rather than ossified. Storey also demonstrated that the effects of cortisone on cartilaginous growth in rabbits were that the long columns of metaphysseal cartilage were replaced by a layer of new bone, partly sealing the epiphyseal cartilage from the medullary canal.

Bone changes which have been described as a result of cortisone therapy lead one to suspect concomitant changes in alkaline-phosphatase activity. It was the object of this investigation to determine whether such changes occur.

MATERIALS AND METHODS

Eight adult male and female mongrel dogs, weighing from ten to twelve kilograms, were used in the experiments. The animals were divided into two groups: four control dogs and four cortisone-treated dogs. The cortisone-treated animals received an initial intramuscular injection of 100 milligrams, followed by fifty milligrams daily of compound E (the average dose per kilogram of body weight was two to four milligrams per day) for four weeks after operation.

A rectangular graft of cortical bone (two by three centimeters) was removed from the upper half of the shaft of each tibia and transplanted to the opposite leg. Two weeks after the first operation, a large rectangular area, which incorporated the previous healing transplant, was removed from the left tibia for tissue study. The right tibia was similarly operated on four weeks after the first operation. Sixteen specimens were available for the histological and histochemical studies. Roentgenograms were made of each leg prior to each operation.

Freshly removed surgical specimens were cut into smaller pieces to ensure proper penetration of the fixing agent. The samples were fixed immediately in 80 per cent alcohol for eight hours and decalcified by the Lorch method, a citrate-hydrochloric acid buffer of pH 4.5 being employed. The decalcified tissues were routinely dehydrated, embedded with paraffin for two hours at 54 degrees centi-

grade, and cut at eight micra. Tissue sections were stained routinely with Harris hematoxylin and eosin for histological studies and by the revised cobalt method of Gomori 11,12,13 for alkaline-phosphatase activity. A sodium beta-glycerophosphate substrate of pH 9.5 was used in which the tissue sections were incubated at 37 degrees centigrade for one-quarter, one-half, one, two, four, eight, and sixteen hours, respectively. Enzyme activity was evaluated by employing a minimal staining technique 21. Semiquantitation of alkaline-phosphatase activity was based on visual inspection of numerous sections prepared from each tissue sample available and on the assignment of arbitrary plus values. The difference between values in activity selected was large enough to prevent the possibility of confusion in the proper assignment of plus values.

RESULTS

Control Group

The two-week-postoperative group: Suitable consolidation of the grafts was observed at this stage grossly. Histological sections exhibited the usual findings.
A section similar to that of Fig. 1, revealing the activity and distribution of alkaline phosphatase. The osteogenic layer of the periosteum and the cells of the intertrabecular spaces, especially the osteoblasts, reveal a high enzyme activity, whereas the graft is devoid of alkaline phosphatase activity at this level of detection (modified Gomori cobalt method, × 50).

A photomicrograph of the area of new-bone formation in a dog in the four-week-postoperative group, revealing the usual picture at this time of increased osteoclastic activity in the subperiosteal areas and the remodeling of trabeculae brought about by the invasion of capillaries and osteoblasts from the periosteum (hematoxylin and eosin, × 430).
of incorporation of a dead graft by the host. The abundant trabeculae that were formed are demonstrated in Figure 1. The osteogenic cells and matrix of the periosteal callus revealed a slightly positive reaction for alkaline phosphatase. This reaction was also found in the fibrous layer of the peristemum. The osteogenic layer of the periosteum, on the other hand, showed greater activity. As the distance from the graft junction to the lateral regions of the callus increased, the levels of alkaline phosphatase also increased.

The cellular components of the new cancellous bone forming about the ends of the graft showed some alkaline-phosphatase activity; the bone graft itself, however, was completely negative except for the blood vessels (Fig. 2).

The four-week-postoperative group: At this period there was the usual histological picture of abundant osteoclastic activity and remodeling (Fig. 3). The peristemal callus exhibited an enzyme activity similar to that found in the two-week-postoperative group, but now the undifferentiated mesenchymal cells, which were present at two weeks, had been replaced by cancellous bone. At sites of active resorption and deposition, the osteoblasts were strongly positive, whereas the osteocytes revealed moderate phosphatase activity. The numerous osteoclasts which appeared at this stage were moderately positive for phosphatase activity.

Cortisone-Treated Animals

The two-week-postoperative group: Histological sections revealed a lack of graft incorporation or, at most, only partial incorporation at this stage; this was also observed grossly. Trabecular bone formation appeared to be minimal (Fig. 4). The numerous and characteristic osteoblasts which were found surrounding the trabeculae (Fig. 3) in the control group were encountered only occasionally in the cortisone-treated dogs. The sparse cellular population of these intertrabecular spaces appeared to be made up primarily of fibroblastic elements. Dense fibrous connective tissue appeared to have infiltrated the osteogenic layer of the periosteum. Only occasional small foci of new-bone formation were seen at the periphery of the graft.
Alkaline-phosphatase activity was greatly reduced throughout all the tissue sections in the cortisone-treated animal (Fig. 5). This was easily noted by gross

**Fig. 5**

A section similar to Fig. 4, studied for alkaline-phosphatase activity and distribution. Note the drastic reduction in enzyme activity throughout the photomicrograph and especially the periosteum, which is indicated by the arrow (modified Gomori cobalt method, × 50).

**Fig. 6**

An enlarged photomicrograph of a region taken from a dog treated with cortisone in the four-week postoperative group, showing poorly ossified trabeculae and a lack of sufficient osteoblasts and osteogenic cells making up the intertrabecular spaces. The morphology of the cells usually associated with normal trabecular formation and remodeling is missing (hematoxylin and cosin, × 430).
TABLE I

<table>
<thead>
<tr>
<th>Observation*</th>
<th>Two Weeks after Operation</th>
<th>Four Weeks after Operation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Dogs</td>
<td>Treated Dogs</td>
</tr>
<tr>
<td>Abundance of osteogenic cells</td>
<td>+ + +</td>
<td>+</td>
</tr>
<tr>
<td>Abundance of connective tissue</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Abundance of cartilage</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>General vascularity</td>
<td>+ +</td>
<td>=</td>
</tr>
<tr>
<td>Trabecular formation</td>
<td>+ +</td>
<td>=</td>
</tr>
<tr>
<td>Abundance of osteoblasts lining intertrabecular spaces</td>
<td>+ +</td>
<td>=</td>
</tr>
<tr>
<td>Graft incorporation</td>
<td>+ +</td>
<td>=</td>
</tr>
<tr>
<td>Over-all alkaline-phosphatase activity</td>
<td>+ +</td>
<td>=</td>
</tr>
<tr>
<td>Periosteal alkaline-phosphatase activity</td>
<td>+ +</td>
<td>=</td>
</tr>
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* + + + = high; + + = moderate; + = low; = = very low.

inspection of the slides, before examining them under the microscope.

The four-week-postoperative group: The over-all histological picture in the dogs in the four-week-postoperative group resembled to some extent sections from control animals at about two weeks after surgery. The tibial grafts which were now in the process of incorporation revealed increased trabecular formation. Active osteoblasts were rarely encountered about the surfaces of the trabeculae (Fig. 6).

Alkaline-phosphatase activity at this stage was only slightly elevated over that in the dogs in the two-week-postoperative group treated with cortisone.

A comparison of the findings in the groups at two weeks and four weeks after operation is given in Table I.

DISCUSSION

The healing of autogenous tibial grafts in dogs treated with cortisone lagged behind that in the control animals by about two weeks. This retardation is in agreement with the results obtained in other animals by several investigators. 7,20. The retardation of appositional periosteal growth was well demonstrated by the presence of "reversal" lines in rabbits treated intermittently with cortisone. 20. Duthie and Barker demonstrated in roentgenograms of the fractured tibiae of cortisone-treated rats that calcium salts of poor density were laid down. These authors believed that the calcium salts were deposited in the free chondroitin sulphate at the fracture site so that calcification rather than ossification was taking place. Although the roentgenograms indicated that fracture healing was taking place, actual consolidation of the fracture site by new-bone formation had not occurred. We have also observed this lack of graft incorporation in all cortisone-treated animals two weeks after surgery. It is interesting to note that newly formed trabeculae in the animals treated with cortisone in the four-week-postoperative group stained lightly with eosin, rather than with the deep red stain that occurs with properly ossified trabeculae. Even more striking was the absence of the large active osteoblasts, which are usually seen surrounding the newly formed trabeculae, and the sparse cellular complement of the intertrabecular spaces. The abundance of trabeculae normally seen two weeks after fracture was not encountered until four weeks after operation. Grossly, grafts from the cortisone-treated animals were not fixed to the surrounding bone two weeks after surgery. They could be removed easily from the defect. The lack of incorporation was clearly shown in the roentgenograms. True incorporation did not occur until four weeks after trauma.
The new bone forming about the graft of the cortisone-treated dogs exhibited a very low alkaline-phosphatase activity. This finding suggests that cortisone treatment with an adequate dosage during bone repair produces diminished alkaline-phosphatase activity. The important normal participation of this enzyme in bone formation is also reduced, and may be associated with the retardation of fracture healing. Normally, one expects an over-all increase in alkaline-phosphatase activity at the fracture site. The association of phosphatase activity with bone formation is well known 1,8,9,10. It was shown by Bourne 3,4,5 that within twenty-four hours after trauma to the femora and skulls of guinea pigs, the periosteum about the injured area exhibited intense phosphatase activity. Tonna 22 further showed that injury to a rat's femur, even in old age, resulted in an elevation of alkaline-phosphatase activity. Trauma to the bone during cortisone therapy, however, was associated with a fall in phosphatase activity below the level of non-traumatized animals not receiving cortisone.

It appears from the results obtained in dogs, that heavy dosages of corticosteroids do have some early loosening effect on the transplants, although healing still did occur. We have found that cortisone used in doses of up to 1,000 milligrams per day for months in a patient with pemphigus produced profound and rapid demineralization in the spine and in the humeral and femoral heads. Such so-called cortisone-produced osteoporosis has been observed in other conditions also—notably, in rheumatoid arthritis after long-term treatment with corticosteroids and in Cushing's disease.

Since cortisone evidently reduced the alkaline-phosphatase activity at the sites of trauma in this series of dogs as well as in rats, it seems probable that so-called cortisone osteoporosis may be effected through this mechanism in the human being. No evidence exists as yet, however, to prove this. We have observed a number of fractures in patients receiving cortisone therapy in dosages comparable to those given to these dogs. All these fractures healed uneventfully.

SUMMARY

Cortisone treatment of dogs after transplants of autogenous tibial grafts revealed sharply reduced alkaline-phosphatase activity two and four weeks after surgery. Graft incorporation and suitable trabecular formation were not seen until four weeks after transplantation in corticosteroid-treated dogs; the trabeculae of the callus lacked the usual osteoblastic lining. Histologically and grossly, the graft site in cortisone-treated dogs exhibited a retardation in bone healing of two weeks when compared with that in control dogs.

REFERENCES