BIOCHEMICAL AND HISTOCHEMICAL CHANGES IN MUCOPOLYSACCHARIDES DURING HEALING OF AN EXPERIMENTAL FRACTURE

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In studies of regeneration during recent years much attention has been paid to the mucopolysaccharides. The synthesis of mucopolysaccharides is known to be one of the links in the morphogenesis of the fibrous proteins of the connective tissue [7, 8, 12].

Workers who have studied the mucopolysaccharides of bone tissue have concluded that the role of these substances in the regeneration of bone is to take part in the formation and binding together of the fibrous structures, in the formation of the matrix of the bone tissue, and in the fixation of calcium [3, 4, 6, 7].

However, the biological role of the mucopolysaccharides in the regeneration of bone has not yet been adequately explained. One of the defects of the studies which have been made of this problem is the absence of any clear connection between the data concerning the chemical and the morphological structure of regenerating bone tissue.

A combined investigation of the mucopolysaccharides of regenerating bone could shed light on some of the principles governing the regeneration of this type of connective tissue and help to explain this process from a wider biological aspect.

The object of the present investigation was to study the general dynamics of the mucopolysaccharides of developing callus and of the peripheral blood. By means of histochemical methods an attempt was made to differentiate the acid mucopolysaccharides and to define their correlation with the morphological structures at each stage of regeneration.

EXPERIMENTAL METHOD

Experiments were conducted on 88 rabbits of both sexes, weighing 2500-3000 g. In sterile conditions, under morphine anesthesia, an open fracture of the middle third of the diaphysis of the right radius was produced in the animals. The bone was not immobilized after the operation, but the ulna acted as a physiological splint. The rabbits were kept in identical conditions before and after the operation.

The stages of regeneration were defined by roentgenological and histological methods, and the chemical characteristics of the stages were established biochemically and histochemically. Observations were made 7, 14, 21, 30, 45, and 60 days after the operation. Because the morphology of regeneration of rabbit bone after fractures has been adequately described [2, 5], this aspect will not be discussed.

The total content of mucopolysaccharides of the regenerating bone was determined by the method of Elson and Morgan, as modified by Boas [10], from the sum of the amino sugars. The serum mucopolysaccharides (glycoproteins) were investigated by Rimington's method [11], based on the preceding method. The corresponding bones and serum of the intact animals were used as controls. A statistical analysis was made of the results of the biochemical investigations.

The histochemical investigations of the acid mucopolysaccharides in the regenerating bone were made by staining with toluidine blue (metachromasia test), with alcian blue, and with colloidal iron by Hale's method. The control methods included treatment of the sections with testicular and bacterial hyaluronidase, repetition of the metachromasia reaction at pH<4.0, methylation and demethylation by the method of Spicer and Lillie [16], and preliminary sulfatation by the method of Moore and Schoenberg [15].

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Content of Mucopolysaccharides in Intact and Regenerating Bone Tissue and in the Peripheral Blood (in mg%)

Statistical index	Intact bone	Bone fragments	Callus						Peripheral blood		
			7 days	14 days	21 days	30 days	45 days	60 days	before fracture	7 days	30 days
n	10	5	8	8	8	8	7	5	11	11	11
M	156	167	415	400	344	189	182	170	87	160	108
$\pm m$	4	13	36	32	32	11	18	13	5,7	4,8	5,7
P	—	0,5	0,001	0,5	0,2	0,02	0,5	0,5 0,5 [1]	—	0,001	0,01 0,01

Note. The mucopolysaccharides of the regenerating bone were expressed in mg% of the dry, defatted tissue.

*In relation to the intact bone.

EXPERIMENTAL RESULTS

The results of the biochemical investigations are given in the table.

As the table shows, the content of mucopolysaccharides in the bone fragments 7 days after the fracture corresponded to their level in the intact bone tissue. Marked changes were found in the mucopolysaccharides of the regenerating bone. The concentration of these compounds was highest 7 and 14 days after the fracture. At the beginning of the third week a tendency for it to fall appeared. By the end of the first month the decrease in the mucoplysaccharide content was significent, and by 60 days it was almost at its original level. The regenerating periosteal tissue was investigated separately 7 and 14 days after the fracture. The mucopolysaccharide content in this case was many times higher than in the callus as a whole (after 7 days 1300±39 mg%, after 14 days 1160±48 mg%, $P = 0.05$). The content of mucopolysaccharides in the serum was significantly elevated 7 days after the fracture. By the end of the first month their level was still above its preoperative value.

Histochemical investigation of 24 animals showed that the content of acid mucopolysaccharides in the callus increased 7 days after the fracture, remained high 14 days after, and then decreased. By the 60th day of regeneration the topographic histochemistry of these substances was essentially the same as in normal mature bone.

At each stage of regeneration the largest accumulations of acid mucopolysaccharides were observed in the ground substance of the chondroid tissue, in the zones of proliferation and differentiation of the osteogenic tissue, at the sites of formation of the matrix of the trabeculae, and in the fibrous tissue structures (periosteum, endosteum, mucle fibers). During the development of the callus the mucopolysaccharides moved from the periphery to the center, i.e., toward the younger zones. A clue to the source of synthesis of these biological compounds in the young zones of the callus was given by the increased basophilia of the cytoplasm of the fibroblasts and osteogenic cells. Seven and 14 days after the fracture considerable vascular and perivascular accumulations of mucopolysaccharides were seen at the sites of differentiation of the osteogenic cells.

The control methods of investigation showed that on the seventh day the callus tissues were rich in hyaluronic acid (incubation with bacterial hyaluronidase). A small amount was still found 14 days after the fracture. The presence of nonsulfated mucopolysaccharides in the first stages of regeneration was also confirmed by the results of methylation of the sections (with restoration of much of the staining after demethylation). Histochemical analysis of the acid mucopolysaccharides in the regenerating bone showed that the sulfated mucopolysaccharides were distributed mainly in the ground substance of the cambial layer of the periosteum, in the chondroid tissue, at the sites of formation of the trabeculae, and in the matrix of these structures. After the ossification of these structures no acid mucopolysaccharides could be detected in them histochemically. Among the acid sulfated mucopolysaccharides, subtances of the chondroitin sulfate C type predominated at first, but later, with maturation of the bone structures, the content of keratosulfate and chondroitin sulfate A increased. Neither chondroitin sulfate B nor heparin are found in bone tissue [14].

Experiments with preliminary sulfatation of the sections and sulfatation after treatment with bacterial hyaluronidase revealed a marked increase in the intensity of metachromasia on staining with toluidine

blue and by other reactions in the tissues of the seven-day callus. This was evidently connected with the presence of compounds of the chondroitin type, precursors of the sulfated mucopolysaccharides [1].

The results of the biochemical investigation of the callus corresponded in the main to data reported in the literature [3, 9, 13] and to the histochemical findings.

The high values discovered on biochemical analysis of the isolated subperiosteal tissues corresponded to histochemical observations showing the selective accumulation of mucopolysaccharides in the functionally most active parts of the regenerating bone (the sites of proliferation and differentiation of the osteogenic mesenchyme, of formation of the matrix of the trabeculae, etc.), and also in the chondroid tissue. The lower indices obtained during the study of the callus as a whole were evidently caused by the inclusion of other tissues containing much smaller amounts of mucopolysaccharides (fragments of muscles, blood clots, bone fragments, etc.). Because of the purpose of the investigation it was judged more correct to investigate the mucopolysaccharides of the callus including all the tissues composing it. Another possibility is that, unlike other investigators [3, 4], we were unable to extract a high enough proportion of the mucopolysaccharides from the tissues, thereby accounting for the difference in the results obtained.

It may be concluded from the biochemical and histochemical data indicating no change in the mucopolysaccharides of the original bone fragments that, although resorption takes place, the composition of the mucopolysaccharides in the residual parts of the fragments remains unchanged.

As was mentioned above, the young bone cells were the source of the mucopolysaccharides in the callus. Comparison between the biochemical data, showing the increase in the mucopolysaccharides in the blood at the height of regeneration, with the histochemical evidence of vascular and perivascular accumulations of these substances suggests that they may also have been brought by the blood.

The process of regeneration of bone tissue is thus accompanied by a considerable change in the content of mucopolysaccharides both in the zone of regeneration and in the peripheral blood. The dynamics of the changes in the content of mucopolysaccharides in the callus and blood quantitatively reflects the principal stages of regeneration. The histochemical picture of the general dynamics of the mucopolysaccharides in the regenerating bone reflects the changes in these substances qualitatively.

It is concluded that determination of the content of mucopolysaccharides in the callus and peripheral blood, and comparison of the results with histochemical findings, can permit a wider interpretation of the activity of the regenerative processes and their possible outcome.

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