COLLAGENASE AND PROTEINASE INDUCTION IN IMPLANTS OF BONE MATRIX

Basil S. Strates* and Marshall R. Urist

Bone Research Laboratory, School of Medicine, University of California, Los Angeles, California 90024

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Summary: Enzymes capable of digesting collagen and non-collagenous proteins are present in implants of bone matrix. In the early stages of bone morphogenesis, implants produce relatively large amounts of trypsin-labile proteins and have high non-collagenolytic neutral proteinase and low collagenase activities. Enzymatic activity is maximal three weeks after implantation. The results indicate that increased synthesis of non-collagenous proteins and non-collagenolytic proteinases precedes the induction of significant amounts of collagenases. The importance of these findings in bone morphogenesis is discussed.

Acid demineralized compact bone induces the formation of new bone when implanted into muscle (1,2). The implants begin to calcify about 10 days after implantation and have a calcium content of 12-14% of dry weight after 32 days (3,4). Induction of alkaline phosphatase occurs as early as 5-6 days and the activity of the enzyme reaches peak values after 20 days (3). Under the influence of a calcification inhibitor, ethane-1-hydroxy-1,1-diphosphonate (EHDP), implants fail to calcify but initial and maximal activity values for alkaline phosphatase are the same as in control animals (4).

Collagenases have been shown to be present in rat, mouse and human skin, human gingiva and rheumatoid synovium, mouse and human bone (5) and in human skin extracts after separation from inhibitors (6). Protein hydrolases are widely distributed in animal tissues (7). Ryan and Woessner (8) recently reported measurements

*Present address: Departments of Medicine and Biochemistry, University of Southern California, Los Angeles, California 90033.

of collagenolytic activity in the involuted uterus after extraction of inhibitors by homogenization in cold 0.9% NaCl solutions and incubation of the residue in the presence of calcium ions, EDTA and trypsin. That report provided us with a tool for studying the induction of collagenases and also of non-collagenolytic neutral proteinases in implants of bone matrices.

MATERIALS AND METHODS

Bones from young adult Sprague-Dawley rats were prepared for implantation as previously described (1-3). Femurs and tibias were demineralized in dilute HCl at 4°C for 2 days and then washed several times with water. Mid-shafts were separated, cleaned of non-osseous tissue, washed and freeze-dried. The demineralized and freeze dried bone matrices were implanted into abdominal wall muscles of rats of the same strain and age as the donor animals. Thirty-two rats were implanted with 8 implants of bone matrix each. At intervals of 5, 10, 21 and 32 days after implantation 8 host animals were sacrificed, abdominal walls excised and X-rayed and representative implants examined histologically for new bone (1). The remaining implants were cleaned from adhering extraneous tissues. Eight implants from each time interval were dried at 110°C overnight, hydrolyzed in 6 M HCl at 125°C for 18 hours in an autoclave and analyzed for calcium by atomic absorption spectrophotometry (9). Since the demineralized bone matrix contains less than 1.5 mg calcium/g of dry weight and only newly formed bone calcifies after implantation (3,4), the calcium content of the implants was used as a measurement of bone morphogenetic activity. Forty-eight implants from each interval were pooled, minced and homogenized with a Vir-Tis homogenizer at medium speed for 10 minutes in cold 0.9% NaCl. Homogenates were centrifuged at 6,000 g and residues incubated for 48 hours at 37°C in 0.04 M Tris buffer, pH 7.5, buffer with 0.01 M

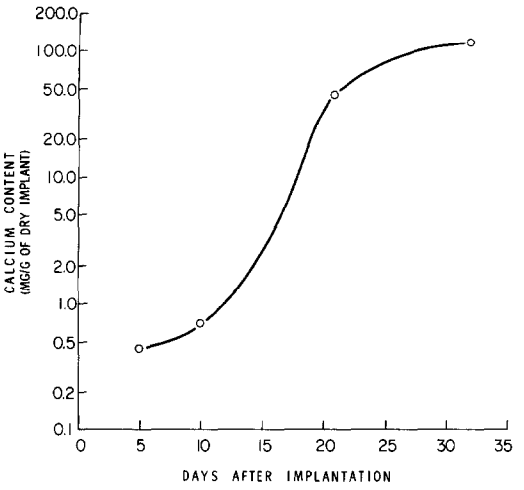

Fig. 1.

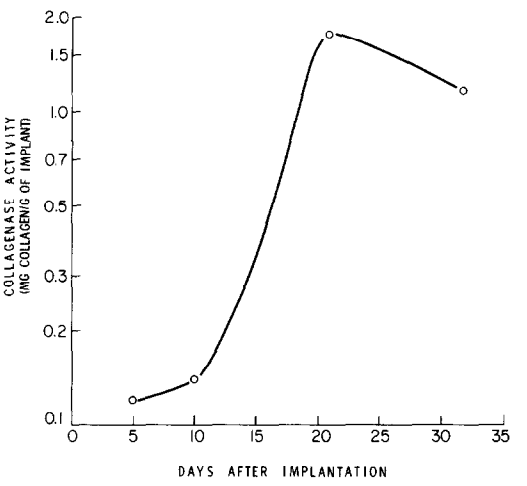

Fig. 2.

Fig. 1. Calcium content of implants 5-32 days after implantation. Implants were cleaned from adhering tissues, dried at 110°C, hydrolyzed in 6 M HCl and analyzed for calcium (9). Points are average values from 6 samples of 2 implants each.

Fig. 2. Collagenase activity in bone implants as a function of time after implantation. Implants were minced and homogenized in cold 0.9% NaCl and homogenates centrifuged to remove collagenase inhibitors (6). Tissue residues were incubated in buffer, pH 7.5, for 48 hours at 37°C and controls at 2°C. Incubations were carried out in the presence of Ca^{++}, trypsin and EDTA. Collagenase activity (mg hydroxyproline X 7.46 = mg collagen) was calculated from the difference between buffer containing Ca^{++} and buffer containing EDTA. Corrections were made for hydroxyproline removed at 2°C and by the buffer at 37°C.

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calcium acetate, buffer with 0.001 M EDTA and 0.01% trypsin or buffer with 0.01% trypsin. Control residues were incubated at 2°C. Penicillin (200 U/ml) and streptomycin (250 g/ml) were added to prevent bacterial growth. After incubation, suspensions were chilled and centrifuged for 30 minutes at 30,000 g (6). Supernates were freeze-dried, hydrolyzed and analyzed for hydroxyproline (10) and amino acids (11).

RESULTS AND DISCUSSION

The calcium content of the implants at various time intervals

after implantation is presented in Fig. 1. As expected (3,4), the 5-day old implants were roentgenographically negative for mineral and had a calcium content approximately equal to that of the unimplanted bone matrix preparations. Most 10-day old implants had roentgenographically detectable mineral deposits and more than twice as much calcium as the original matrix. Calcium content rose rapidly to about 50 mg/g of dry implant weight 21 days after implantation and limit values of about 120 mg/g were reached after 32 days.

Collagenase activity in the 5- and 10-day old implants was low, rose abruptly to a maximum in implants 21 days after implantation and declined appreciably in the 32-day old implants (Fig.2). In the interval of 5-21 days after implantation collagenase activity increased as the mineral content of the implants increased. After 21 days, mineral content continued to increase whereas the activity of the enzyme was decreased.

In Fig. 3 are presented data on the induction of non-colagenolytic neutral proteinases and on the levels of non-collagenous proteins removed from the implants after incubation with trypsin. Proteinase activity increased rapidly to peak values 21 days after implantation and decreased slowly thereafter. Peak amounts of proteins susceptible to trypsin hydrolysis were removed from implants 10 days after implantation but their concentration decreased rapidly to values lower than those from the 5-day old implants. For the interval 10-21 days after implantation neutral proteinase activity was inversely proportional to the amount of proteins removed by trypsin.

Collagenases are required for bone growth and remodelling (5, 8) and proteinases active at neutral pH have been recently assigned a role in bone morphogenesis (7). The induction of these enzymes in implants of bone matrix follows a course described by curves

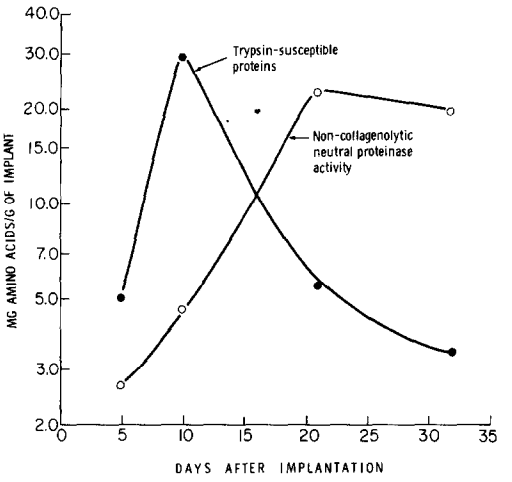

Fig. 3. Proteinase (neutral, non-collagenolytic) activity and levels of trypsin-susceptible proteins in bone implants 5-32 days after implantation. Conditions were the same as in Fig. 2. Proteinase activity was calculated from the difference between total amino acids and hydroxyproline after incubation in the presence of EDTA. Non-collagenous proteins were calculated from the difference between total amino acids and hydroxyproline after incubation with trypsin and EDTA. Corrections were made for amino acids and hydroxyproline removed at 2°C (controls) and by the buffer at 37°C.

which are similar to those published by Schimke (12) for mammalian enzyme induction systems. The low levels of collagenolytic activity in the early stages of bone morphogenesis may reflect a low population of collagenase synthesizing cells. The subsequent abrupt increase in collagenolytic activity may, on the other hand, indicate a rapid rise in the number of collagenase producing cells or an increased rate of differentiation of primitive (mesenchymal) cells into fibro- and osteoblasts. The observed drop in collagenase activity in later stages of bone morphogenesis may suggest further differentiation of cells into osteocytes.

The quantitative relationships established by the present study between neutral proteolytic activity and protein removal by trypsin from implants of bone matrix show that the induction of

significant amounts of proteinases is clearly preceded by the synthesis of large quantities of trypsin-labile proteins. This and published observations that incubation of matrices with trypsin before implantation produces tissue residues devoid of bone morphogenetic activity (13,14), an effect not shared by glycolytic enzymes and nucleases (7), implicate trypsin-labile non-collagenous proteins in bone morphogenesis.

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