Idenification, Distribution, and Significance of a Collagenolytic Enzyme in Human Tissues.* (31702)

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An enzyme capable of splitting collagen molecules had not been demonstrated in human tissues when this investigation was begun. Although surgeons have long realized that such an enzyme must exist, attempts to demonstrate collagenolytic activity in human tissue by preparing various tissue homogenates and extracts and testing their ability to lower the viscosity of collagen solutions have failed to reveal decisive and reproducible cleavage of the collagen molecule. Though enzymes such as cathepsin, papain, and pepsin have a measurable effect on collagen (particularly immature collagen), their effect is so small or requires such harsh unphysiologic substrate conditions (low pH, gelatinization temperatures, etc.) that they can hardly be considered seriously as agents responsible for the rapid removal of large amounts of collagen in conditions such as maturing scars, spreading trophic ulcers, or involution of a gravid uterus (4,8,9,10).

In 1963 it occurred to Gross and Lapiere that the enzyme responsible for collagenolysis under physiological conditions might be attached to the living cells and could be demonstrated only by growing tissue specimens on collagen substrate under carefully controlled conditions. When tissue from the rapidly involuting dorsal fin of a metamorphosing tadpole was cultured on reconstituted collagen which had been dialyzed against amphibian Tyrode's solution, lysis of the substrate occurred as long as cells in the implant remained alive(6). Subsequently, Gross and his colleagues have been able to extract and purify a few milligrams of the enzyme from tadpole tissue and have been able to show that it attacks the tropocollagen molecule by cleaving the main polypeptide chain approximately two-fifths of the way along its longitudinal axis; kinetic studies of this enzyme are being made in Gross's laboratory(2,7). In the meantime, Grillo, using the Gross tissue culture technique, has identified collagenolytic enzyme in the margin of contracting skin wounds in guinea pigs(5). During preparation of this manuscript, collagenolytic activity in normal and diseased human gingival tissue was discovered by a similar technique (1,3).

The objectives of our investigation have been to determine whether or not a collagenolytic enzyme could be identified in human beings and, if so, under what conditions and in what tissues it could be measured by tissue culture techniques.

Materials and methods. Collagen was obtained for tissue culture plates by soaking fresh calf hide for 3 days at room temperature in 5% ammonium chloride containing 1% sodium chloride and 1:10,000 merthiolate so that the epidermis could be separated and the subcutaneous tissue removed. The clean dermis was washed repeatedly with cold water and then minced coarsely with large scissors. The dermal fragments were extracted with 10 volumes of .5 molar acetic acid for 24 hours with constant stirring at 5°C, following which the highly viscous extract was passed successively through bolting cloth and then coase and fine sintered glass filters. The acid solution of monomeric collagen was lyophilized and stored at —20°C.

Collagen solutions were prepared by dissolving 60 mg of lyophilized collagen in 15 ml of cold phosphate buffer (7/2 = .4, pH 7.6). Approximately 12 hours of constant mechanical shaking was needed to get the collagen into solution. The phosphate-collagen solution was then dialyzed against several changes of .4 molar sodium chloride for 24 hours, and the resulting sodium chloride solution of monomeric collagen was passed through sterile glass filters and stored in sterile serum bottles at 5°C.

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Culture plates were prepared by adding one part of sterile neutral collagen solution to 3 parts of synthetic mammaliam Tyrode's solution to which penicillin and streptomycin were added. Approximately 2 cc of the collagen-Tyrode's mixture were transferred to 30 mm sterile tissue culture dishes and incubated at 37°C for 6 hours. Heat gelation of the neutral collagen produces a cloudy gel upon which various tissue fragments can be implanted. Cultures were prepared under sterile conditions by implanting 3 × 3 mm fragments of tissue on the surface of the collagen gel; the covered plates were then incubated at 37°C in a 10% CO_2 atmosphere. Lysis of the gel by collagenolytic activity of the tissue produced a clear zone around the implant which, in most specimens, continued to enlarge until the entire plate had undergone complete lysis. All positive plates were subcultured anaerobically on thioglycollate broth to be certain that they were not contaminated with clostridial organisms.

Tissue from the involuting tail of a thyroxin treated *Rana catesbeiana* (giant leopard bull frog) tadpole, or rat uterus slices were used as positive controls to check tissue culture conditions before cultures were classified as negative. Although many of the plates became contaminated with pathogenic bacteria (Staphylococci, Streptococci, and various Gram-negative rods), collagenolysis was never observed around colonies of bacteria other than clostridia.

Two hundred thirty-eight specimens of human tissues from normal and pathological organs were removed from patients in the operating rooms of the North Carolina Memorial Hospital. Specimens were transferred immediately to a flask of mammalian Tyrode's solution and were placed in an incubator until they could be implanted on collagen substrate approximately one hour later. All plates were examined each day after implantation and after 5 days negative plates were discarded.

Results. The results obtained from culturing 238 specimens of normal and abnormal human tissue are shown in Table I-V. Although negative cultures have been recorded, we cannot state with certainty that any tissue positively does not elaborate collagenolytic enzyme. With the exception of wound margin and granulation tissue, it is rare to find collagenolytic activity in all fragments of a single tissue specimen; in evaluating negative results, therefore, it must be remembered that demonstration of collagenolytic activity is dependent upon keeping tissue alive in an artificial medium for 5 days. Cell death results in instantaneous cessation of enzyme activity, and the failure of many tissue fragments to produce collagenolytic enzyme could be failure of tissue culture methods to sustain life for 5 days. In addition, many tissues which have the capability of elaborating collagenolytic enzyme may not do so as a steady state phenomenon but may be pulsed to produce enzyme at specific intervals or in response to specific stimuli which have not been identified. Other variables such as time of separation of tissue from blood supply, plane of implantation, and unrecognized fluctuations in tissue culture conditions

TABLE I. Skin and Healing Wounds of Skin.

Tissue	Collagenolysis	
	Positive	Negative
Normal skin	17	12
Wound margin	10	0
Granulation	8	0
Eschar	3	3
Healed scar (including 3 Keloids which were positive)	23	9
	61	24

TABLE II. Mesenchymal Tissue and Deep Scar.

Tissue	Collagenolysis	
	Positive	Negative
Tendon	0	14
Tendon scar and adhesions	0	14
Fascia (including Dupuytren's)	0	15
Nerve and neuroma	1	8
Muscle	0	3
Artery and aneurysm	0	5
Joint capsule and synovium (normal and rheumatoid)	0	10
Bone	0	6
Fracture callus	0	3
Intra-abdominal scar	0	9
Myometrium	0	6
Spleen	1	2
Liver	0	3
Lung	0	3
Kidney	0	3
	2	104

TABLE III. Gastrointestinal Tract.

Tissue	Collagenolysis Positive	Negative
Oral mucosa	1	1
Esophageal mucosa	0	1
Gastric mucosa	1	2
Gastric ulcer (benign)	1	2
Duodenal mucosa	1	2
Duodenal ulcer	1	0
Colonic mucosa	1	6
Colonic ulcer (chronic ulcer colitis)	1	0
	7	14

TABLE IV. Neoplasms.

Type	Collagenolysis Positive	Negative
Cutaneous	7	3
Breast	0	3
Prostate	1	0
Larynx	0	1
Cervix	2	2
Ovary	1	0
Colon	1	0
Gastric	1	0
Sarcoma	0	2
Fibroma	0	2
	13	13

TABLE V. Collagenolytic Activity of 238 Human Tissues.

Tissue type	Collagenolysis Positive	Negative
130 tissues with epithelium	81	49
108 tissues without epithelium	2	106

undoubtedly have produced many negative results which otherwise might have been positive.

Normal skin from all age groups (premature infants to 75-year-old adults) produced both positive and negative cultures (Table I). Cultures of cutaneous scar tissue were found to be positive as early as 14 days after wounding and as long as 30 years following injury. Human scar less than 14 days old was not available for culture. The 10 positive specimens of wound margins reported in Table I were taken from wounds which varied between 8 and 36 days of age. Specimens from human wounds less than 8 days old were not tested. Scar tissue from rat skin wounds does not show collagenolytic activity, however, before the eleventh day after wounding. Granulation tissue from the center of human wounds is positive for collagenolytic activity as soon as granulations appear (5 days). Third degree burn eschar was negative for approximately 2 weeks after injury, at which time it uniformly became strongly positive. Clinical evidence of separation of the eschar was usually associated with laboratory measurement of collagenolytic activity.

Table II reveals the almost complete lack of any demonstrable collagenolytic activity in deep tissues which do not contain epithelial cells. One neuroma which was positive is of questionable significance because of its superficial location in cutaneous scar. The only clear-cut example of deep mesenchymal tissue collagenolytic activity is one specimen of an enlarged spleen from a patient with idiopathic thrombocytopenic purpura. Failure to find evidence of enzyme activity in remodeling scar tissue around old tendon repairs and healing bone callus is difficult to explain, as is the failure to find lytic activity in tissue specimens from strategic places in rheumatoid hands where gross collagen destruction in collateral ligaments, long tendons, and the extensor hood had altered normal architecture in grotesque fashion. Perhaps the lytic phase of the process was burned out and arthritic joint tissue must be examined earlier if abnormal collagenolytic enzyme activity is to be found.

Ulcerations of the gastrointestinal tract appear to be associated with collagenolytic activity, which raises the interesting question of whether activation or suppression of enzyme is important in the production and control of these lesions (Table III). Epithelial tumors also produce collagenolytic enzyme and the importance of lytic activity in such processes as invasion and metastasis must be investigated (Table IV).

Discussion. The most important hypothesis concerning the site of collagenolytic enzyme production which these data suggest is the apparent correlation of enzyme activity with the presence of epithelial cells. Failure to find the enzyme in mesenchymal tissues where obvious collagenolysis and collagen remodeling can be observed may be the result of inadequate tissue culture conditions for mesen-

chymal tissues, or may simply mean that there are other mechanisms of removing collagen which presently are not measurable by tissue culture techniques. Grillo separated epithelium from underlying dermis and mesenchymal tissue in healing wounds of guinea pigs. He found that only epithelium produced lysis but that more lysis occurred when epithelium and mesenchymal tissue were cultured together(5).

A significant number of human ailments are characterized by abnormally large amounts of collagenous tissue, and a few ailments are caused by a deficiency of collagen. Discovery of an enzyme in human tissue which has the ability to lyse collagen molecules suggests the feasibility of looking at such conditions from the standpoint of an imbalance between collagen synthesis and breakdown. The relatively crude methods which show that degradation and synthesis are occurring simultaneously, however, may not be sensitive enough to measure minute differences in enzyme kinetics which, over a long period of time, could account for gross tissue abnormalities. Moreover, the problem of knowing precisely when to look for small abnormalities may be critical, as there is a growing body of evidence to indicate that synthesis is not a continuous process. If this is so, the search for dynamic abnormalities will have to be carefully timed or imbalances which are physiological at certain times could be misinterpreted as pathological, and significant imbalances at crucial times could be overlooked because samples were collected at inappropriate intervals.

Summary and conclusions. 1. Collagenolytic activity in normal and pathological human tissue has been demonstrated by culturing tissue specimens on reconstituted bovine collagen substrate. 2. Collagenolysis appears to be associated primarily with epithelium and not with deep connective tissue elements. 3. Alteration of a delicate balance between collagen synthesis and collagen breakdown in remodeling tissues has been hypothesized as the mechanism by which some pathological conditions characterized by abnormal amounts of collagen may be produced.

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Effect of Acetoacetate and β-Hydroxybutyrate on Vitamin B_{12} in Rats. (31703)

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The intermediary compounds involved in fat metabolism, acetoacetate and β-hydroxybutyrate on prolonged administration have been shown to cause increase in blood sugar (1), blood lactic and pyruvic acids(2,3), and blood urea and inorganic phosphate(4). These ketone bodies also showed increased cholesterol/phospholipid ratio(5), disturbances in the balance of hepatic glycogenesis and glycogenolysis(6), decrease in reduced