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Collagenase and corneal ulcers

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Collagen is the major organic component of the cornea. It composes more than 60 per cent of the dry weight of the tissue.[1]

The collagen molecule has a molecular weight of 300,000 and is composed of three helical polypeptide chains which are wound around a common axis forming a coiled chain.[2] In solution, the collagen molecules exist as long rods about 3,000 × 15 Å, have a high intrinsic viscosity, and exhibit a strongly negative optical rotation. When the temperature and pH of a solution of collagen approach 37° C. and 7, respectively, the collagen molecules polymerize into insoluble fibrils. Accordingly, it is as fibrils that collagen almost invariably exists in tissue. Collagen, either in solution or as fibrils, may be denatured by elevated temperatures or exposure to high concentrations of reagents such as calcium chloride.[3] Associated with denaturing or uncoiling of the molecule is a decrease in the intrinsic viscosity and optical rotation of solutions of collagen.

The helical structure of undenatured collagen in solution or fibrils is remarkably resistant to attack by proteolytic enzymes. Trypsin, chymotrypsin, and pepsin may in time solubilize collagen fibrils by attacking the polar portions of the molecules or bonds between adjacent molecules, but they do not change the

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helical structure of the molecules.[4] Accordingly, these enzymes do not change the characteristic intrinsic viscosity or optical rotation. However, if collagen is denatured, the uncoiling of the molecule exposes more bonds which could then be attacked by the proteolytic enzymes.

A collagenase derived from *Clostridium histolyticum* was the first known enzyme that could attack the helical backbone of the collagen. It attacks and attaches to the molecule from either end and does not release its hold until the polypeptide chains are reduced almost to amino acids.[5]

It has long been suspected that an enzyme is produced in animal tissues which could remove collagenous tissue. Until relatively recently, all efforts at isolating such an enzyme were fruitless. However, in the last ten years, intensive studies on the acid hydrolases and the isolation and characterization of a neutral collagenase have clarified the mechanisms of collagen degradation in animal tissues.

Lysosomal enzymes

Acid hydrolases, which are usually of lysosomal origins, can degrade collagen, but only at a pH below 6.0. Consequently, it is doubtful that these enzymes can operate extracellularly to have any effect on collagen fibrils under physiologic conditions. The main function of the proteolytic acid hydrolases in connective tissues is generally considered to be associated with intracellular activity where the pH is acidic and the hydrolases can digest collagen debris as part of the process of phagocytosis.[6]

Collagenase

In 1962, Gross and Lapiere[7] reported the first of a series of studies which demonstrated a collagenolytic enzyme in tissues that could operate under physiologic conditions. Collagenolytic activity was demonstrated and assayed by culturing tissue explants on opaque collagen gels. Lysis or clearing of the gel indicated collagen breakdown. The collagen, which was heat gelled, was essentially fibrillar and provided a sensitive assay system when labeled with ^{14}C. The first studies were on the metamorphosing tadpole tail and showed that collagenase was produced mainly in the epithelial cells but also in the underlying mesenchyme. Collagenolytic activity was also observed in mammalian skin wounds.[8] The greatest enzyme activity appeared to originate from the epithelium, although there was some contribution from the underlying granulation tissue. Collagenolytic activity was detected in other tissues, i.e., skin,[8] bone,[9] the involuting uterus,[10] and inflamed gingiva.[11]

Characteristically, neither homogenates nor extractions of these tissues produced collagenolytic activity and freeze-thawing of the tissue explants stopped enzyme production. This indicated that collagenase was produced by living cells and was not stored in the tissues, at least in detectable quantities.[7] The collagenases produced by the metamorphosing tadpole tail,[12] by mammalian skin,[8] and by the arthritic joint[13] were harvested and characterized. In contrast to the almost total breakdown of the collagen molecules by bacterial collagenase, these enzymes cleaved the collagen molecule across the helical backbone yielding ¾ and ¼ length fragments. This limited cleavage reduced the viscosity of collagen solutions by 40 per cent but did not alter the negative optical rotation. It is recognized that this type of cleavage, the viscosity change, and the unaltered optical rotation are rather characteristic of all animal collagenases. Also characteristic are the inhibitors of collagenase. Dilute solutions of cysteine, ethylenediaminetetraacetic acid (EDTA), and serum inhibit almost all the collagenases.[9] An exception is the collagenase of the postpartum rat uterus which is not inhibited by cysteine.[10] How these substances inhibit animal collagenase has not been studied. It is generally assumed that the activity of the collagenases is dependent on calcium ions. This was found to be true for the collagenase of the postpartum rat uterus.[10] It is possible

that chelators like the EDTAs inhibit by removing calcium ions from the environment of collagenase. The dynamics of inhibition by serum and cysteine are understood even less. A recent study showed that the globulin fraction of serum could inhibit collagenase.[14]

Recently, two collagenases have been reported that deserve separate mention. The first is found in granules of the granulocytes and as such is recognized after destruction of the cell membranes rather than by tissue culture. It is interesting that, although the granulocyte collagenase can cleave collagen in solution, it has limited activity on collagen fibrils—the state in which collagen exists in tissue.[15, 16]

The second collagenase was isolated from the synovial fluid of the rheumatoid arthritic joint. The cell origin of this enzyme is unknown. This enzyme from the synovial fluid is similar to granulocyte collagenase because it is not inhibited by serum, but it differs in that it has greater activity against collagen fibrils.[17]

Collagenase and the cornea

Recently, Brown and associates,[18] with the use of a slight modification of the tissue culture–assay technique of Gross and Lapiere,[7] found that ulcerated tissues of the alkali-burned cornea show collagenolytic activity. This was confirmed by Gnädinger and co-workers[19] whose tissue culture assay incorporated the use of a Rose chamber.

Collagenase was determined to be the cause of the ulcers of the alkali-burned cornea when it was shown that the harvested enzyme from a single ulcer produced an immediate ulcer when injected into an intact alkali-burned rabbit cornea[20, 21] and that the usually inevitable perforations could be prevented by topical applications of collagenase inhibitors.[22, 23]

Brown and Weller[24] showed that cysteine was the most efficacious in preventing the ulcers, NaEDTA was less effective and irritating, and CaEDTA was ineffective.[24] However, Itoi and colleagues,[23] in a study of less extensively burned rabbit corneas, reported that CaEDTA applied more frequently prevented the ulcers and perforations.

Characteristics of corneal collagenase

The enzyme produced by the ulcerated corneas in tissue culture was harvested from the tissue culture medium, partially characterized, and shown to be a true collagenase: The enzyme reduced the viscosity of a solution of collagen by 40 to 50 per cent and it did not alter the optical rotation of collagen solutions.[25] The optimum activity of the enzyme occurred at a pH between 7 and 8. The enzyme was completely inactivated in an acid environment.[26] The site of collagen cleavage by corneal collagenase came from a technique utilizing the so-called segment long-spacing (SLS) aggregates. Gross and Nagai[12] showed that collagen molecules will precipitate from acid solutions in the presence of adenosine triphosphate so that the molecules are aligned in perfect register. The length of these SLS aggregates is the approximate length of the molecule (3,000 Å). The effect of corneal collagenase on the length of the SLS aggregates was observed with the electron microscope; the collagen molecule was cleaved into the rather characteristic ¼ and ¾ length fragments.[26]

Dynamics of collagenase inhibitors

In a study of the dynamics of collagenase inhibitors, Weller and Brown[26] found that corneal collagenase is dependent on calcium for its activity. They then showed that isolated corneal collagenase is inhibited by dilute concentrations of Na-EDTA, $CaNa_2EDTA$, cysteine, and serum. The EDTA preparations, which are known powerful chelators, probably inhibit by removing the essential calcium. This is proved by the findings of Weller and Brown[26] which showed that addition of calcium to a combination of EDTA and corneal collagenase reactivated the enzyme and reversed the inhibition of EDTA.

Dialyzing EDTA from the combination also reactivated the enzyme. These studies demonstrate that the EDTAs are reversible inhibitors that act indirectly by removing calcium from ions from the environment of the calcium-dependent collagenase.[26]

Cysteine is known to be a weaker chelator and there is some evidence indicating that part of its inhibition of corneal collagenase may be due to chelation of calcium.[26] Of greater therapeutic importance is the fact that cysteine has been shown to inhibit corneal collagenase irreversibly indicating that it acts by directly attaching to and inactivating the collagenase molecule. This type of inhibition approaches the ideal from a therapeutic standpoint.[26] Furthermore, the reversible inhibition by the EDTAs may explain the difference in the studies of Itoi and colleagues[23] and Brown and co-workers.[22] The former showed that $CaNa_2EDTA$ applied hourly prevented ulcer formation, while the latter found $CaNa_2EDTA$ to be ineffectual. Because $CaNa_2EDTA$ is a reversible inhibitor, it is possible that its efficacy depends on more frequent administration.

Cysteine's irreversible inhibition of corneal collagenase is similar to that envisioned for cysteine's inhibition of bacterial collagenase. Harper and Seifter's[27] and Harper's[28] studies indicated that cysteine irreversibly inhibits by attaching to a metal ion on the molecule of bacterial collagenase. Accordingly, 2,3-Dimercaptopropanol (BAL) was shown to be the most powerful inhibitor of bacterial collagenase. Because of the similarities in the mode of cysteine inhibition of both bacterial and corneal collagenases, the efficacy of BAL in the treatment of corneal ulcers is being investigated.

The finding that rabbit serum inhibited rabbit corneal collagenase differs from the observations of Itoi and associates,[29] who studied the normal epithelium of rabbits. The main reason for this difference is probably that Itoi and colleagues[29] used commercially packaged serum, which was found to exhibit only one third of the inhibitory powers of fresh serum.

Recently, Gnädinger and co-workers[19] incubated swollen frozen sections of normal corneas with hyaluronidase and found that more of these stromas lost their ability to take the Giemsa stain when exposed to hyaluronidase. They theorized that reduction of the ground substance by hyaluronidase could make corneal collagen more vulnerable to collagenase. Though there are other ways to interpret these results, the mentioned theory has import. A similar possibility was raised by Brown and associates[21] who showed that the alkali-burned cornea which had lost the majority of its ground substance from exposure to alkali was more susceptible to corneal collagenase than the normal cornea. Studies by Weller and Brown[26] showed that mixing collagen with the proteoglycan extracted from either the cornea or cartilage prevented collagen breakdown by corneal collagenase. However, collagenase activity is not altered by mixing the collagen with purified keratan SO_4, which does not include protein. It was concluded that proteoglycans of the cornea protect collagen from collagenase breakdown by presenting a physical barrier.[26]

Cell origin of corneal collagenase

Itoi and associates[29] studied the normal epithelium of the rabbit cornea and found that it exhibited small amounts of collagenolytic activity. Brown and Weller[30] also assayed the normal rabbit epithelium with both nonlabeled and ^{14}C collagen gels and could not detect collagenolytic activity. Furthermore, neither Brown and Weller[30] nor Slansky and his colleagues[31] could find collagenolytic activity in the human epithelium. Brown and Weller[30] showed that although abraded corneal epithelium did not produce collagenase, significant quantities of collagenase were produced by the epithelium surrounding a penetrating corneal wound. Recently, the author assayed combinations of normal

epithelium and wounded stroma. Separately the tissues did not produce collagenase, but collagenolytic activity was observed when the tissues were cultured together (unpublished results). It was concluded that elements of granulation tissue induce the wounded epithelium to produce collagenase.

Studies on the cell origin of collagenase in the alkali-burned cornea showed that in the immediate vicinity of the ulcer, collagenase was produced by the regrowing epithelium and also by the underlying stroma. In addition, collagenase was produced in the stroma of the more peripheral vascularized intact areas, but not in the epithelium. Stromal explants (without epithelium) elaborated the enzyme even after the cells were killed by repeated freeze-thawing, suggesting a collagenase of a granulocyte origin. However, the stromal enzyme could effectively lyse collagen fibrils, which is not characteristic of granulocyte collagenase.[16, 20]

Recent investigations by Brown and Weller[30] of corneal stromal granulomas also showed a collagenase that was elaborated from stromal tissues that were previously frozen and then thawed repeatedly. Puromycin allowed enzyme production, but reducing the circulating white blood cell count with nitrogen mustard prevented collagenase activity. This suggested a collagenase of granulocytic origin, but again, the stromal enzyme was able to lyse relatively large quantities of collagen fibrils. The stromal enzyme was isolated and characterized and found to be a true collagenase.[32]

Pathogenesis of collagenase-induced tissue destruction

The previously mentioned data allow for speculation as to the pathogenesis of the ulcers of the alkali-burned cornea and other noninfectious corneal ulcers as collagenase has been found in most forms of inflamed corneal ulcers. In the case of the alkali-burned cornea, exposure of the cornea to alkali results in immediate death of all the cells in the cornea, as well as loss of the majority of the proteoglycan of the ground substance.[33, 34] Approximately one week after an alkali burn, the epithelium and stromal vascularization (which is always accompanied by fibroblasts and granulocytes) begin a slow central progression. Collagenase is produced both by the advancing epithelial cells and in the underlying stroma.

In spite of collagenase production and the loss of the protective proteoglycan environment of the stromal collagen, the stroma peripheral to the advancing cells remains intact. This peripheral area is almost always vascularized and it could be that serum proteins diffusing from the relatively permeable new vessels would inhibit the stromal collagenase in the immediate area of the new vessels. In addition, the accelerated production of new collagen by the many activated fibroblasts in the stroma may counterbalance collagenase breakdown. At the advancing cell border, collagenase is produced by the epithelium in addition to stromal cells.

Ulcerations of the stroma central to this advancing border may be explained by collagenase production in an area beyond the limits of the new vessels and where there are few to no fibroblasts. These ulcers are almost never seen before ten days and theoretically, to prevent ulcers, collagenase inhibitors should be started by seven days after the injury. Treatment must be continued until the epithelium has covered the cornea and the epithelial collagenase production has stopped. The stromal production of collagenase continues for a long time after epithelial healing, but does not cause stromal dissolution perhaps because of inhibition of this enzyme by serum proteins.

These studies may also explain wound dissolution after corneal transplantation for alkali burns. When the host epithelium reaches the wound interface, the epithelium is induced to produce epithelium by the underlying stromal wound. For some reason, the epithelium is generally slow to

bridge the wound resulting in prolonged enzyme production and tissue dissolution.

The method of collagenase destruction in corneal ulcers of eyes not burned with alkali is probably slightly different and involves selective enzymatic breakdown. If an epithelial defect is associated with stromal inflammation, the epithelium is induced to produce collagenase. Inflammatory enzymes produced in the stroma may hydrolyze the proteoglycan protection of the stromal collagen, and following this, the epithelial collagenase can break down the stroma. Ulcers seem to heal when new blood vessels reach the inflamed area. The author speculates that further ulceration is stopped by the leaking of serum proteins from the new vessel which inhibits collagenase and stops further collagen breakdown.

Clinical studies with collagenase inhibitors

No matter the type of corneal ulcer, one must be cautious of coincident treatment with corticosteroids. Brown and colleagues[25] found that corticosteroids potentiate collagenase activity by 15x. This may be the reason that certain ulcers rapidly progress to perforation after treatment with corticosteroids.

Presently, clinical studies on the treatment of collagenase-induced corneal ulcers have been rather limited to the treatment of the various problems associated with the alkali-burned cornea.[20, 21] Brown and Weller[24] reported rather preliminary studies indicating that perforations of alkali-burned human corneas may be prevented by topical treatment with cysteine (0.2M). They also suggested treatment with collagenase inhibitors as an adjunct to corneal transplantation to prevent wound breakdown in the postoperative course.

More recently, Brown and Weller treated 26 eyes with various problems associated with the alkali-burned cornea. Despite the notoriously poor prognosis of the conditions treated, all eyes responded favorably, i.e., there was no loss of corneal substance. The authors treated 11 other eyes with various types of corneal ulcers or healing problems. All eyes eventually healed without loss of substance. They concluded that the uniformly successful treatment of the alkali-burned eyes warranted the use of collagenase inhibitors for the various problems associated with alkali burns. The value of treatment of other types of corneal ulcers though suggested was not proved.[35]

It is obvious that much work is still to be done in this new field, but these summarized studies indicate that collagenase-induced tissue destruction exists and that collagenase inhibitors may play a prominent role in the treatment of diseases of the cornea.

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