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TYPE-SPECIFIC COLLAGENOLYSIS: A TYPE V COLLAGEN-DEGRADING ENZYME FROM MACROPHAGES

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SUMMARY: An enzymatic activity capable of degrading type V collagen at neutral pH was found in the medium from cultured rabbit pulmonary alveolar macrophages which had been "activated" in vivo by injection of complete Freund's Adjuvant. This enzyme was characterized as a metalloproteinase by virtue of its inhibition by EDTA but not by phenylmethylsulfonyl fluoride or N-ethyl maleimide. Ion-exchange chromatography on DEAE-cellulose was successful in separating the type V collagen-degrading activity from the type I collagenase which is also secreted by these cells. These observations suggest that the degradation of type V collagen is independent of the degradation of the interstitial collagens and may require the action of its own "specific collagenase."

The animal collagenases (EC 3.4.24.7) represent a relatively homogenous group of metalloproteinases which can be synthesized by a variety of mesenchymal cells. Despite subtle differences among some of these enzymes, they all function by making a single cleavage through the triple helix yielding two fragments, TC^A and TC^B (1). It is this specific cleavage which is felt to be essential in the initiation of collagenolysis in physiologic remodelling of tissues and in pathologic states.

At present, there are at least five known genetically distinct collagens differing not only in chemical composition but also in tissue distribution and, to some degree, susceptibility to enzymatic degradation. At neutral pH, types I and II collagen are only cleaved by the classic collagenases. Type III collagen, although also cleaved by these enzymes, possesses peptide bonds in the same region of the molecule which are susceptible to trypsin (2), thermolysin (3), and granulocyte elastase (4,5). Type IV or basement membrane collagen is resistant to the action of classical collagenases (6) but is cleaved by several other proteinases

Abbreviations Used: PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; NEM, N-ethylmaleimide

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(7,8,9). There is little known about the degradation of type V collagen although it has been shown to be resistant to the action of rheumatoid synovial collagenase (01) and undergoes limited proteolysis when incubated with trypsin (8).

In the present study, it is shown that "activated" pulmonary alveolar macrophages secrete an enzyme which is capable of degrading type V collagen. This enzyme appeared to be a metalloproteinase but could be separated from the "classical" or interstitial collagenase by ion-exchange chromatography. It is also shown that the type V collagen-degrading enzyme was inactive against type I collagen and the "classical" or collagenase did not cleave type V collagen at 27^0C.

METHODS

Preparation of Cell Cultures: New Zealand White rabbits (2-2.5 kg) were injected with 0.2 ml of complete Freund's Adjuvant into an ear vein according to the method of Horwitz and Crystal (11) in order to produce an "in vivo activation" of alveolar macrophages. The animals were sacrificed 17-20 days after the injection by the parental administration of a sublethal dose of Ketamine followed by an intravenous injection of a bolus of air. The lungs were removed surgically and lavaged with phosphate buffered saline without calcium and magnesium (PBS)[1] (GIBCO) (50 ml/lavage x 8). The cells were pelleted by centrifugation and the cell pellets washed x 3 with PBS. The cells were finally resuspended in Dulbecco's Modified Eagle's medium (GIBCO) and plated out in 15 x 100 mm Petri dishes (Falcon) at 10^7 cells/dish. The cells were cultured at 37^0C in an atmosphere of 95% air/5% CO_2 and the medium was harvested and changed every 48 hrs until the cultures were no longer viable (10-30 days). The medium was pooled and kept at -20^0C. The medium was thawed and concentrated 50-fold by pressure dialysis (Amicon).

Preparation of Collagens: Type I collagen was prepared from acid extract of calf skin and type III collagen was prepared from pepsin extract of human cirrhotic liver using estabilished methods of purification (12,13). Type IV collagen was prepared from 0.5 M acetic acid extract of bovine anterior lens capsule. The collagen was purified by adjusting the NaCl concentration to 0.6 M, collecting the precipitate by centrifugation, redissolving in 0.5 M acetic acid and precipitating the native collagen at neutral pH by thermal gelation as described by Schwartz et al (14). Type V collagen was prepared from pepsin extracts of human placentae according to the method of Rhodes and Miller (15). This was further purified by TCA-ethanol precipitation according to the method of Gross (16).

Enzyme Assays: Types I and V collagen were radiolabelled using ^{14}C acetic anhydride according to Cawston and Barrett (17). Degradation of type I collagen was detected by using reconstituted collagen gels according to Nagai et al (18). Type V collagenolysis was detected using a modification of the method devised by Terato et al for type I collagen (19). 50 ul of ^{14}C type V collagen was incubated for 4 hr at 32.5°C with 50 ul of sample in a volume of 150 ul with a final concentration of 0.04 M Tris-HCl/0.5 M NaCl/5 mM $CaCl_2$, pH 7.6. The reaction was stopped by the addition of EDTA to 5 mM and the undigested collagen precipitated by the addition of dioxane to 50% v/v. The mixture was allowed to stand 4 min, was centrifuged, and an aliquot of the supernatant counted in a liquid scintillation counter in 10 ml of Aquasol (New England Nuclear).

Collagenolytic activity detected by the above assays was confirmed by electrophoretic identification of the reaction products. Soluble collagen was incubated with enzyme solution in 0.04 M Tris-HCl/0.15 M NaCl/5 mM $CaCl_2$ at 27°C for 16 hrs.

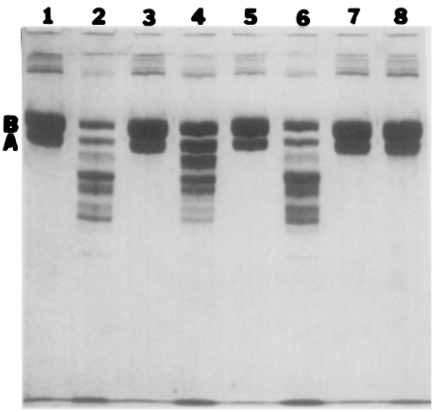

Figure 1. Degradation of type V collagen by pulmonary alveolar macrophage (PAM) culture medium. 100 µg purified type V collagen was incubated at 32.5°C with 100 µl of culture medium. Controls were incubated with buffer alone. Track 1 is control collagen, track 2 is collagen + enzyme, track 3 is collagen + PMSF (1 mM), track 4 is collagen + enzyme + PMSF, track 5 is collagen + N-ethyl maleimide (NEM) (5 mM), track 6 is collagen + enzyme + NEM, track 7 is collagen + EDTA (5 mM) and track 8 is collagen + enzyme + EDTA. As can be seen, the enzyme activity was totally inhibited by EDTA but not by NEM or PMSF. On this basis we have characterized this enzyme as a metalloprotease.

L-arginine was added to each determination to prevent fibril formation (0.05 M). The reaction was stopped with EDTA and the samples electrophoresed on a slab gel apparatus (Hoeffer) according to the method of Laemmli (20). Types I and V collagen were run without a reducing agent while types III and IV collagen were run in the presence of 2-mercaptoethanol.

Elastase activity was detected against ^{3}H elastin according to Gordon et al (21). SDS was not included in the reaction mixture.

DEAE Cellulose Chromatography: DE-52 (Whatman) was packed in a column (1 x 25 cm) and equilibrated with 0.05 M Tris-HCl/5 mM $CaCl_2$/0.02% NaN_3, pH 8.3 at 4^0C. The sample was applied with a flow rate of 56 ml/hr, 19 ml fractions were collected, and the column washed with the starting buffer until the absorbance at 280 nm returned to baseline. The bound proteins were eluted in a linear gradient of 0-0.15 M NaCl in the same buffer. The total gradient volume was 1000 ml.

RESULTS

When type V collagen was incubated at 32.5^0C for 15 hrs with crude concentrated macrophage medium, collagenolysis could be observed by SDS gel electrophoresis (Fig. 1). This activity was inhibited by EDTA but not by N-ethyl maleimide (NEM) or phenylmethylsulfonyl fluoride (PMSF). It can also be seen that the reaction products were of large (> 60,000) molecular weight despite the long incubation. This was particularly surprising in view of the likelihood that other contaminating proteinases may have been present in this crude material.

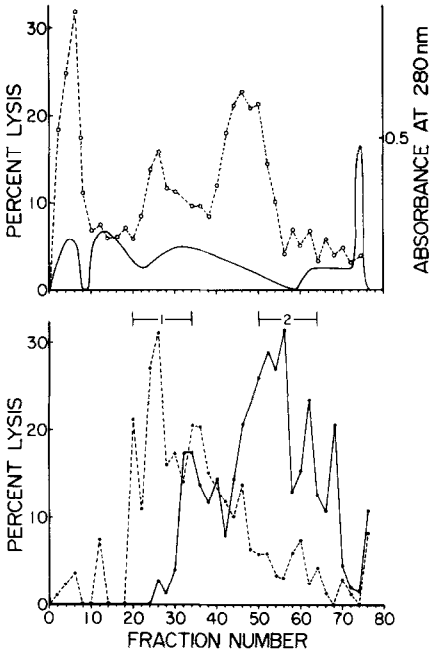

Figure 2. DEAE cellulose chromatography of crude macrophage culture medium. 84 ml of crude culture medium was applied to the column. The gradient was begun at fraction 9 and 1 M NaCl was applied at fraction 70. In the upper panel, the absorbance at 280 nm is represented by the solid line and the elastase activity is indicated by the open circles, broken line. In the lower panel, the type I collagenolytic activity is shown by the closed circles, broken line and the type V collagen degrading activity is indicated by the closed circles, solid line. The fractions which were pooled and concentrated are indicated by the brackets.

It has been well established that these cells are capable of secreting a collagenase of the "classical" variety (11,12). Thus, we sought to determine if the type V collagen degrading activity was due to the same enzyme. When the crude culture medium was applied to a column of DEAE-cellulose and the proteins eluted in a shallow gradient of NaCl, the two activities were clearly separated (Fig. 2). As can be seen in the lower panel, a small amount of the type I collagen-degrading activity passed through the resin without binding but most eluted in the NaCl gradient. In contrast, all the type V collagen-degrading activity adhered to the resin and eluted at higher NaCl concentrations. A large amount of elastase activity was found in the void peak while some eluted in the gradient. These observations strongly suggest that these three proteolytic activities were the result of at

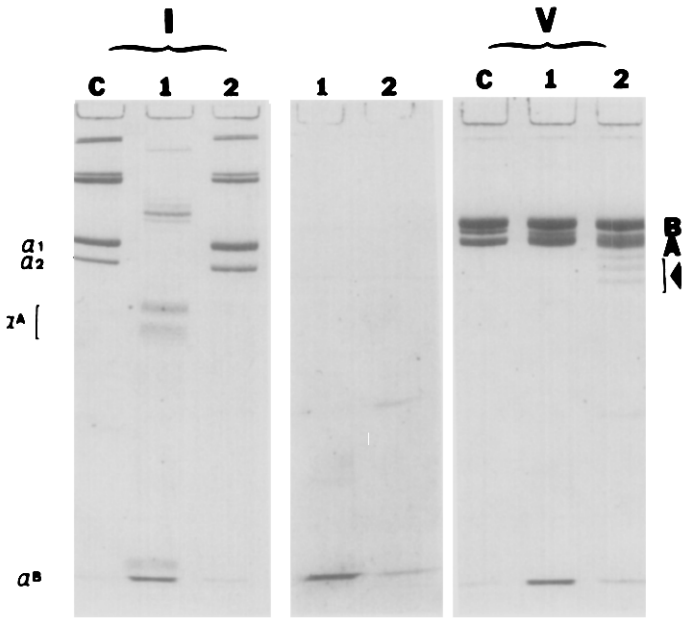

Figure 3. Identification of the reaction products of types I and V collagens on 7.5% acrylamide gels. The brackets above the gels indicate the collagen type. C represents collagen incubated at 27°C for 16 hr with buffer alone. 1 indicates collagen incubated with pool 1 and 2 indicates collagen incubated with pool 2. The 2 tracks in the center represent pools 1 and 2 respectively incubated without collagen. When type I collagen was incubated with pool 1, the classic reaction products, α^A and α^B are seen. Pool 2 had no effect on type I collagen. However, when type V collagen was incubated with pool 2, three distinct reaction products can be seen (arrow) while pool 1 had no effect on type V collagen.

least three separate enzymes but it cannot be excluded that the type V collagen degrading enzyme has some elastolytic effect.

It was noted that the enzyme activity in each of the peak fractions lysed only 30% of the substrate in 16 hrs. This was due largely to the dilution caused by such a large elution volume. To confirm these observations, we pooled the fractions which represented the peak of type I collagen degradation (pool 1) and type V collagen degradation (pool 2). Each pool was concentrated 15-fold by pressure dialysis and incubated for 16 hrs at 27°C with types I, III, IV, and V collagens as described. In Figure 3 it is seen that pool 1 completely degraded type I collagen but had no effect on type V collagen, whereas pool 2 had no effect on type I collagen but three larger molecular weight reaction products were generated by pool 2. (It should be noted that the type V collagen degradation proceeded slowly at 27°C

and only the larger reaction products are observed. This temperature was chosen to identify the reaction products of type I collagen which might have been further degraded by other proteinases after denaturation at higher temperatures.) Type III collagen was completely degraded by pool 1 while pool 2 caused very little degradation of this collagen. Type IV collagen was partially degraded by both pools (data not shown). The degradation of these latter two collagens by macrophages may involve other enzymes and these problems are presently under investigation.

DISCUSSION

It has been shown that cultured rabbit pulmonary alveolar macrophages release an enzyme which is capable of degrading type V collagen. This enzyme appears to be a metalloproteinase and is different from the "classical" collagenase secreted by these cells. There has been little published on the degradation of this unusual collagen. We have shown in a previous report that purified rheumatoid synovial collagenase did not degrade type V collagen (10). Liotta and his colleagues have shown that trypsin caused limited cleavage of the molecule (8) and have also demonstrated that culture medium from a murine reticulum cell sarcoma cell line and human leiomyosarcoma cell strain contained a type V collagenolytic enzyme (personal communication).

It can be concluded from these data that the macrophage-mediated degradation of type V collagen in the rabbit lung is enzymatically independent of the degradation of type I collagen. It is also possible that the rate of release under different conditions into the extracellular space as well as their inhibition by naturally occurring proteinase inhibitors are also different. Thus, it can be hypothesized that, under certain pathologic conditions, an imbalance of collagen types could develop by the process of selective depletion of the matrix of a specific collagen type. This may play a role in pathologic conditions of organs such as lungs where up to 10% of the pepsin-extracted collagen is type V (Seyer, J.M., unpublished data).

In recent years, our understanding of the connective tissue matrix has expanded to include novel collagen types. Similarly, our understanding of the matrix must expand to accomodate the differences in proteinase susceptibility of these various collagens. In addition to the differences already mentioned, it has been shown that type III collagen is resistant to granulocyte collagenase (22) but is cleaved by granulocyte elastase (5). All these findings are likely to cause confusion in the nomenclature of the collagenolytic enzymes. Thus, it might be proposed that the "classical collagenases" be referred to as "interstitial collagenases" because they degrade the interstitial collagens. Until further studies are completed, the enzymes which degrade types IV and V collagens should be referred to in terms of their respective substrates.

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REFERENCES

1. Harris, E. D., Jr., and Cartwright, E. C. (1977) in Proteinases in Mammalian Cells and Tissues (Barrett, A. J., ed) pp. 249-284
2. Miller, E. J., Finch, J. E., Jr., Chung, E., Butler, W. T., and Robertson, P. B. (1976) Arch. Biochem. Biophys. 173, 631-637
3. Wang, H. M., Chan, J., Pettigrew, D. W., and Sodek, J. (1978) Biochim. Biophys. Acta 533, 270-277
4. Mainardi, C. L., Hasty, D., Seyer, J. M., and Kang, A. H. (1980) J. Biol. Chem. (in press)
5. Gadek, J. E., Fells, G. A., Wright, D. G., and Crystal, R. G. (1980) Biochem. Biophys. Res. Comm. 95, 1815-1822
6. Woolley, D. E., Glanville, R. W., Roberts, D. R., and Evanson, J. M. (1978) Biochem. J. 169, 265-276
7. Mainardi, C. L., Dixit, S. N., and Kang, A. H. (1980) J. Biol. Chem. 255, 5435-5441
8. Liotta, L. A., Abe, S., Robey, P. G., and Martin, G. R. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2268-2272
9. Uitto, V-J., Schwartz, D., and Veis, A. (1980) Eur. J. Biochem. 109, 409-417
10. Chiang, T. M., Mainardi, C. L., Seyer, J. M., and Kang, A. H. (1980) J. Lab. Clin. Med. 95, 99-107
11. Horwitz, A. L., and Crystal, R. G. (1976) Biochem. Biophys. Res. Comm. 69, 296-303
12. Glimcher, M. J., Francois, C. J., Richards, L., and Krane, S. M. (1964) Biochem Biophys. Acta 93, 585-602
13. Seyer, J. M., and Kang, A. H. (1977) Biochemistry 16, 1158-1164
14. Schwartz, D., Chin-quee, T., and Veis, A. (1980) Eur. J. Biochem. 103, 21-27
15. Rhodes, R. K., and Miller, E. J. (1978) Biochemistry 17, 3442-3448
16. Gross, J. (1958) J. Exp. Med. 107, 247-277
17. Cawston, T. E., and Barrett, A. H. (1979) Anal. Biochem. 99, 340-345

18. Nagai, Y., Lapière C., and Gross, J. (1966) Biochemistry 5, 3123-3130
19. Terato, K., Nagai, Y., Kawaniski, K., and Yamamoto, S. (1976) Biochim. Biophys. Acta 445, 753-762
20. Laemmli, U. K. (1970) Nature 227, 680-685
21. Gordon, S., Werb, Z., and Cohn, Z. A (1976) in In Vitro Methods in Cell Mediated and Tumor Immunity (Bloom, B. R., and David, J. R., eds.) pp. 341-352, Academic Press, New York
22. Horwitz, A. L., Kelman, J. A., and Crystal, R. A. (1976) Nature 264, 772-774
23. Horwitz, A. L., Hance, A. H., and Crystal, R. G. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 879-901