Reports

Oxidative Autoactivation of Latent Collagenase by Human Neutrophils

Abstract. *The pathological destruction of collagen plays a key role in the development of inflammatory disease states affecting every organ system in the human body. Neutrophils localized at inflammatory sites can potentially degrade collagen by releasing a metalloenzyme, collagenase, which is stored in a latent inactive form. Triggered human neutrophils were shown to release and simultaneously activate their latent collagenase. The activation of the latent enzyme was coupled to an oxidative process that required the generation of a highly reactive oxygen metabolite, hypochlorous acid. Oxidative regulation of latent collagenase activity may be important in the pathogenesis of connective tissue damage in vivo.*

Collagen is the major structural protein in the human body, controlling tissue architecture, cell growth, and cell differentiation (*1*). The destruction of this key connective tissue component during inflammation is important in the pathogenesis of a host of disease states affecting every organ system in the body (*2*). One of the hallmarks of acute inflammatory damage is the influx of large numbers of blood neutrophils into the affected site (*3*). The neutrophil can potentially degrade collagen by releasing the lysosomal metalloenzyme, collagenase, which is stored in the specific granules in a latent, inactive form (*2, 4*). However, once the enzyme is released into the extracellular milieu, it must be activated in order to catalyze collagen degradation (*2, 4*). Despite intense interest in delineating the role of collagenases in the development of pathologic tissue damage, neither the ability of the human neutrophil to activate the latent enzyme nor the processes involved in the control of the proteinase have been characterized (*2, 4*). We have now shown that (i) triggered neutrophils simultaneously release and activate large amounts of their latent collagenase and (ii) collagenase activation is controlled by an unusual oxygen-dependent process.

Human neutrophils were isolated from venous blood by a Ficoll-Hypaque separation followed by dextran sedimentation (*5*). Neutrophils (10^7 per milliliter) were suspended in Hanks buffered salt solution (Gibco) at *p*H 7.4 and triggered by the addition of serum-opsonized zymosan particles (Sigma) or phorbol myristate acetate (PMA) (Consolidated Midland) (*5*). The mixtures were incubated for 15 minutes at 37°C and then centrifuged (1250*g* for 5 minutes). A portion (0.5 ml) of the supernatant was removed and treated with an equal volume of the serine proteinase inhibitor phenylmethylsulfonyl fluoride (2 m*M*) (*6*). Portions (0.2 ml) of this mixture were then examined for collagenolytic activity either by quantitating the release of radioactivity from reconstituted [^{3}H]acetylated type I collagen fibrils at *p*H 7.6 and 37°C (*7*) or by electrophoretic analysis of the products formed after incubation of the supernatants with soluble type I collagen at *p*H 7.6 and 25°C (*4*). Total collagenase activity (that is, spontaneously active and latent enzyme) was determined after the addition of the activating agent 4-aminophenylmercuric acid (APMA; 0.5 m*M*) (*4, 7*).

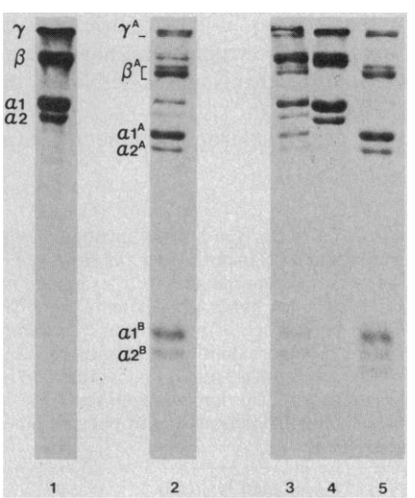

Fig. 1. Proteolysis of soluble type I collagen by neutrophil collagenase. Supernatants from PMA- or zymosan-stimulated neutrophils were prepared as in Table 1 and incubated with ^{3}H-labeled soluble type I collagen alone, or with the agents listed below, for 20 hours at 25°C. Samples were reduced, heat-denatured and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis for which a 3 percent stacking and 8.5 percent resolving gel were used (*15*). Collagen was incubated alone (lane 1), with supernatants obtained from PMA-stimulated cells (lane 2), or with supernatants obtained from zymosan-stimulated cells (lane 3). Collagen was also incubated with supernatants from PMA-stimulated cells treated with EDTA (lane 4), or APMA (lane 5). Products were made visible by fluorography.

Although the specific collagenase is stored in a latent form (*2, 4*), neutrophils triggered with zymosan particles or PMA released significant quantities of active collagenase (Table 1). The addition of APMA to these supernatants activated the remaining latent collagenase and revealed that, of the total collagenolytic activity released by the neutrophils, 41 percent was present in an active form with zymosan as the stimulus, and 63 percent was present with PMA as the stimulus (Table 1). Resting cells did not release significant collagenase activity (0.9 ± 1.8 percent of the collagen was degraded; $n = 5$) but small amounts were detected after the addition of APMA (8.6 ± 2.6 percent; $n = 5$). In order to confirm that the collagen was being specifically degraded by the calcium-requiring collagenase, we incubated the supernatants with radiolabeled, soluble type I collagen in the absence or presence of ethylenediaminetetraacetic acid (EDTA) (10 m*M*) and examined the cleavage products by gel electrophoresis. Mammalian collagenases make only one proteolytic scission in each polypeptide chain of native type I collagen, giving rise to 3/4 and 1/4-size fragments (*2, 4*). As shown in Fig. 1, supernatants obtained from PMA or zymosan-triggered neutrophils (i) cleaved the type I collagen dimers (β1,1 and β1,2) and monomers (α_1, α_2) into the expected 3/4- (β1,1^A, β1,2^A, $\alpha_1{}^A$, $\alpha_2{}^A$) and 1/4- ($\alpha_1{}^B$, $\alpha_2{}^B$) fragments, and (ii) required calcium for their activity (Fig. 1). Thus, triggered neutrophils have the ability to activate their latent collagenase.

In considering the processes that might allow the neutrophil to regulate collagenase activity, we focused our attention on the known ability of thiol-reactive reagents to activate the latent enzyme directly or indirectly (*2, 4, 8*). Triggered neutrophils generate a family of reactive oxygen metabolites including superoxide anion ($O_2{\cdot}^-$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and the hydroxyl radical (OH·), and each of these species has the ability to oxidize thiol-containing compounds

(*9*). The possibility that these oxidants are involved in the activation of latent collagenase was examined by triggering neutrophils in the presence of enzymes known to lower the concentration of $O_2^{\cdot -}$ (superoxide dismutase) or H_2O_2 (catalase). Superoxide dismutase did not alter the amount of active collagenase detected in the supernatant or the total amount of released activity (Table 1). In contrast, the addition of catalase (but not heat-inactivated catalase) to the triggered cells almost completely suppressed the activation of the latent collagenase as assessed in the fibril assay (Table 1) or the soluble collagen system (Fig. 2). Catalase did not interfere with latent collagenase release or the expression of collagenase activity because the addition of APMA to the supernatant unmasked collagenolytic activity (see Table 1). The ability of catalase to inhibit the appearance of active collagenase without interfering with latent collagenase activity indicates that H_2O_2 plays a pivotal role in the autoactivation process.

If H_2O_2 generation is required for collagenase activation, neutrophils obtained from individuals with chronic granulomatous disease (CGD), a genetic disorder characterized by the inability of neutrophils to generate oxygen metabolites (*9*), would be expected to release the latent collagenase but be unable to activate the enzyme. Indeed, the triggered CGD neutrophils failed to express significant collagenolytic activity unless

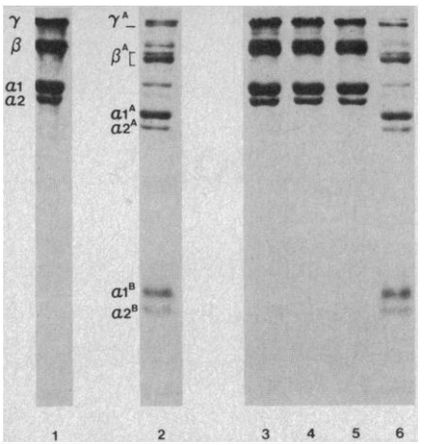

Fig. 2. Expression of specific collagenolytic activity by triggered neutrophils. Soluble ^{3}H-labeled collagen was incubated as in Fig. 1 either alone (lane 1) or with the supernatant obtained from neutrophils incubated with PMA (lane 2), PMA and 25 μg of catalase (lane 3), PMA and 1 m*M* azide (lane 4), PMA and 5 m*M* methionine (lane 5), or PMA and catalase followed by 25 nmol of HOCl (lane 6).

APMA was added to the supernatant (Table 1). However, if these neutrophils were stimulated in the presence of an exogenous source of H_2O_2 (a glucose–glucose oxidase system), active collagenase was generated, and the activation process was rendered catalase-sensitive (Table 1). Thus, in the absence of H_2O_2 production, neutrophils did not have alternative mechanisms for activating the collagenase (*10*).

Triggered neutrophils generate large amounts of H_2O_2, but only small quantities of the H_2O_2 diffuse into the extracellular milieu (*11*). Instead, most of the H_2O_2 is consumed by the lysosomal enzyme myeloperoxidase, which generates the powerful oxidant HOCl (*5*, *9*).

$$H_2O_2 + Cl^- + H^+ \xrightarrow{\text{myeloperoxidase}} HOCl + H_2O$$

We determined the role of the myeloperoxidase system in latent collagenase activation by stimulating neutrophils in the presence of azide, a peroxidase inhibitor, or of methionine, an HOCl scavenger (*12*), and examining the supernatants for collagenase activity. Neither azide nor methionine interfered with the neutrophils' ability to generate $O_2^{\cdot -}$ or H_2O_2, but both agents almost completely blocked the appearance of active collagenase (Table 1). The released collagenase remained in the latent form as demonstrated by its complete activation after APMA was added (Table 1). Finally, the ability of these agents to block the activation of the collagenase could be directly confirmed by their ability to inhibit the appearance of the 3/4- and 1/4-size collagen fragments (Fig. 2).

Taken together, these data suggest that HOCl functions as the final oxidant in the activation sequence and that the direct addition of reagent HOCl to the latent collagenase should lead to the expression of collagenolytic activity. Therefore, neutrophils were triggered in the presence of catalase, and the supernatant containing the latent collagenase was removed. Hypochlorous acid (25 nmol) was added to 0.5 ml of the cell-free supernatant, and the mixture was incubated for 15 minutes at 37°C. The mixture was then treated with phenylmethylsulfonyl fluoride and examined for active collagenase. As predicted, the HOCl-treated supernatants and triggered neutrophils degraded the collagen fibrils in comparable amounts [HOCl-activated supernatant degraded 26.6 ± 7.4 percent (n = 7), whereas supernatant alone degraded 5.7 ± 1.1 percent (n = 3), and HOCl-treated buffer had no effect on released radioactivity] and specifically cleaved collagen into the 3/4- and 1/4-size fragments (Fig. 2).

We have shown that neutrophils can directly regulate the activity of their latent collagenase and have provided an example of an intact cell that uses a powerful oxidizing agent to activate a latent enzyme. Although we have stressed the importance of HOCl as the primary oxidant responsible for collagenase activation, neutrophils can also use HOCl to generate a derivative class of long-lived oxidants, the *N*-chloroamines

Table 1. Collagenolytic activity released by neutrophils. Cells (10^7) were incubated with zymosan particles (1.25 mg) or PMA (30 ng) for 15 minutes at 37°C in the absence or presence of the agents listed below. Phenylmethylsulfonyl fluoride–treated supernatants were examined for collagenolytic activity against 50 μg of ^{3}H-labeled type I collagen fibrils (approximately 3×10^4 count/min) after a 20-hour incubation at 37°C (*8*). The percentage of lysis (mean ± standard deviation) was calculated as [counts released by neutrophil supernatant minus counts released by buffer control] divided by [counts released by bacterial collagenase minus counts released by buffer control]. Buffer controls released 8.4 ± 3.2 percent (n = 28), and collagen treated with 10 μg of trypsin released 14.0 ± 1.9 percent (n = 6). CGD neutrophils are neutrophils obtained from individuals with chronic granulomatous disease.

Supernatants obtained from	Collagen lysis (%)		n
	Without APMA	With APMA	
Neutrophils (zymosan)	13.4 ± 6.1	33.1 ± 8.7	11
Neutrophils (PMA)	25.3 ± 7.4	40.0 ± 10.2	28
Neutrophils (PMA) + superoxide dismutase (10 μg)	25.2 ± 7.5	39.4 ± 7.4	4
Neutrophils (PMA) + catalase (25 μg)	4.5 ± 3.5	59.0 ± 14.6	16
Neutrophils (PMA) + heat-inactivated catalase (25 μg)	21.6 ± 4.5	42.5 ± 3.4	3
CGD neutrophils (PMA)	3.6	46.4	2
CGD neutrophils (PMA) + catalase (25 μg)	3.1	47.9	2
CGD neutrophils (PMA) + glucose oxidase (15 mU)	24.2	27.1	2
CGD neutrophils (PMA) + glucose oxidase (15 mU) + catalase (25 μg)	3.2	51.6	2
Neutrophils (PMA) + azide (1 m*M*)	3.4 ± 3.4	56.7 ± 11.3	7
Neutrophils (PMA) + methionine (5 m*M*)	3.7 ± 2.6	70.8 ± 9.9	4

(*13*). However, preliminary studies indicate that chloroamines are inferior to HOCl as activators of the latent enzyme (*14*). All previous reports have emphasized the destructive properties of HOCl (*9*). This species is used by neutrophils to destroy microbes, mammalian cells, inflammatory mediators, and various enzymes (*9*). Indeed, increased quantities of APMA-activated collagenase were recovered when neutrophils were incubated with catalase, azide, or methionine (column 2 in Table 1). Apparently HOCl can either promote or suppress collagenase activity, but in every situation examined, the oxidative activation predominated.

The reaction scheme underlying HOCl-dependent collagenase activation is unknown and is limited by our incomplete understanding of the molecular basis for the enzymes' latency. HOCl may lead to the dissociation of an enzyme-inhibitor complex, the perturbation of the molecular confirmation of a proenzyme, or the activation of a latent collagenase proactivator (*2, 4, 8*). Nonetheless, our studies with the intact neutrophil demonstrate the critical position that HOCl holds in the activation sequence. We conclude that human neutrophils are able to harness the nonspecific reactivity of HOCl by coupling its generation with the activation of an enzyme capable of mediating specific connective tissue damage. Oxidative regulation of neutrophil collagenase and possibly other tissue collagenases may represent an important step in the pathogenesis of inflammation in vivo and a rationale for the design of specific therapeutic interventions.

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References and Notes

1. T. Linsenmayer, in *Cell Biology of the Extracellular Matrix*, E. Hay, Ed. (Plenum, New York, 1982), pp. 5–32; P. Bornstein and H. Sage, *Annu. Rev. Biochem.* **49**, 957 (1980).
2. J. Gross, in *Cell Biology of the Extracellular Matrix*, E. Hay, Ed. (Plenum, New York, 1982), pp. 217–253; Z. Werb, in *Collagen in Health and Disease*, J. Weiss and M. Jayson, Eds. (Churchill-Livingstone, Edinburgh, 1982), pp. 121–134; R. Perez-Tamayo, in *ibid.*, pp. 135–159.
3. P. Ward, Ed., *Handbook of Inflammation* (Elsevier, Amsterdam, 1983), vol. 4.
4. G. Lazarus, R. Brown, J. Daniels, H. Fullmer, *Science* **159**, 1483 (1968). For recent references see, H. Macartney and H. Tschesche, *Eur. J. Biochem.* **130**, 71 (1983); G. Murphy, J. Reynolds, V. Bretz, U. Baggiolini, *Biochem. J.* **203**, 209 (1982); K. Hasty, M. Hibbs, A. Kang, C. Mainardi, *J. Exp. Med.* **159**, 1455 (1984).
5. S. Weiss, R. Klein, A. Slivka, M. Wei, *J. Clin. Invest.* **70**, 598 (1982).
6. Supernatants were treated with phenylmethylsulfonyl fluoride to prevent neutrophil serine proteinases from attacking the nonhelical portions of type I collagen [A. Barrent, *Agents Actions* **8**, 11 (1978)].
7. Type I collagen was extracted from rat tails and labeled by the method of T. Cawston and G. Murphy [*Methods Enzymol.* **80**, 711 (1981)].
8. V. Uitto, H. Turto, A. Huttenen, S. Lindy, J. Uitto, *Biochim. Biophys. Acta* **613**, 168 (1980); H. Macartney and H. Tschesche, *FEBS Lett.* **119**, 327 (1980); H. Nagase, R. Jackson, C. Brinckerhoff, C. Vater, E. Harris, *J. Biol. Chem.* **256**, 11951 (1981); C. Vater, H. Nagase, E. Harris, *ibid.* **258**, 9374 (1983).
9. S. Weiss, in (*3*), pp. 37–87.
10. Latent collagenases could be autoactivated by serine proteinases (*2, 4*), but neutrophils triggered in the presence of catalase were incapable of activating the collagenase during the 15-minute incubation before phenylmethylsulfonyl fluoride was added. Increasing the incubation period to 60 minutes before the addition of phenylmethylsulfonyl fluoride did not significantly change these findings. Plasmin may also activate collagenases (*2, 4*), but neutrophils triggered in the presence of catalase and plasminogen did not activate their collagenase under these conditions (unpublished observation).
11. S. Test and S. Weiss, *J. Biol. Chem.* **259**, 399 (1984).
12. S. Weiss and S. Regiani, *J. Clin. Invest.* **73**, 1297 (1984).
13. S. Weiss, M. Lampert, S. Test, *Science* **222**, 625 (1983); S. Test *et al.*, *J. Clin. Invest.* **74**, 1341 (1984); M. Grisham, M. Jefferson, D. Melton, E. Thomas, *J. Biol. Chem.* **259**, 1040 (1984).
14. Oxidized glutathione disulfide (GSSG) has been reported to be an activator of purified neutrophil collagenase (*4, 9*). Both HOCl and *N*-chloroamines can oxidize glutathione to its disulfide, but 10^7 neutrophils can release no more than 7 nmol of GSSG if all their intracellular glutathione is oxidized and released [see A. Voetman *et al.*, *Blood* **55**, 741 (1980)]. In our system, the addition of 50 nmol of GSSG failed to activate the latent collagenase.
15. U. Laemmli, *Nature (London)* **227**, 680 (1970).
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Induction of DNA Synthesis in Cultured Rat Hepatocytes Through Stimulation of α_1 Adrenoreceptor by Norepinephrine

Abstract. *Addition of norepinephrine to primary cultures of adult rat hepatocytes stimulates the incorporation of [^{3}H]thymidine in a dose-dependent manner. This effect has been observed in serum-free medium containing epidermal growth factor and insulin. Stimulation of DNA synthesis by norepinephrine was strongly antagonized by the α_1-adrenergic antagonist prazosin but not by an α_2 antagonist or by a β-adrenergic blocker. The β agonist isoproterenol did not stimulate significant DNA synthesis. These results indicate that catecholamines interact with the α_1 adrenoreceptor to stimulate DNA synthesis in hepatocytes. Since α_1 receptors are present in most cells, this receptor may be important in cell growth regulation.*

Cell replication does not generally occur in adult mammalian hepatocytes. However, the hepatic parenchyma responds to partial resection with coordinated waves of DNA synthesis. This has made liver regeneration a useful model for the study of stimulated cell growth.

A role for catecholamines in the stimulation of hepatic DNA synthesis after partial hepatectomy has been suggested by many investigators [reviewed in (*1*)]. In general, adrenergic receptor inhibitors suppress DNA synthesis during liver regeneration in vivo, but whether this effect is mediated by α- or β-adrenergic receptors is unclear. Also unknown is whether catecholamines have a direct effect on DNA synthesis or act by indirect stimulation through increased production or release of other growth factors.

Primary cultures of parenchymal hepatocytes of high viability, capable of entering DNA synthesis after appropriate stimulation, have been established. Rat hepatocytes have been cultured in serum-supplemented or serum-free media containing epidermal growth factor (EGF) and insulin (*2, 3*). We now report that the addition of norepinephrine to cultures of rat hepatocytes results in a dose-dependent stimulation of DNA synthesis and that this effect is antagonized by prazosin, which blocks the α_1-adrenergic receptor.

The addition of high concentrations of norepinephrine to hepatocyte cultures stimulates the incorporation of [^{3}H]thymidine into material that is precipitable with trichloroacetic acid (TCA). Incubation of cultures with various doses of norepinephrine generated the dose-response curve in Fig. 1a. To determine whether the incorporation of [^{3}H]thymidine represented a true stimulation of hepatocyte DNA synthesis, we prepared autoradiographs of parallel cultures and determined nuclear labeling indices. The results demonstrate that a dose-response relation exists between norepinephrine concentration and the stimulation of hepatocyte nuclear labeling. At $10^{-4}M$ norepinephrine, the labeling index was approximately 49 percent.

It is conceivable that toxic effects of norepinephrine might generate an apparent dose-response relation by selectively killing unlabeled cells, thereby increasing the labeling index. We therefore directly assayed the survival of the hepatocytes at all of the norepinephrine concentrations on the dose-response curve.