Vol. 85, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 14, 1978 Pages 490-496

SYNTHESIS OF COLLAGENASE AND NEUTRAL PROTEASES BY ARTICULAR CHONDROCYTES: STIMULATION BY A MACROPHAGE-DERIVED FACTOR

Kalindi Deshmukh-Phadke, Michele Lawrence, and Susan Nanda

Lilly Research Laboratories, Eli Lilly and Co.,
Indianapolis, IN 46206

Received September 21,1978

SUMMARY

Rabbit peritoneal macrophages, when incubated in culture with bacterial lipopolysaccharides, release a factor which stimulates the production of collagenase and neutral proteases by chondrocytes. The possible role of these enzymes in cartilage destruction in chronic inflammatory conditions is discussed.

INTRODUCTION

Erosion of articular cartilage and denudation of bone are the end results of the destructive processes occurring in rheumatoid and osteoarthritic joints. Increased production of collagenase by the hypercellular synovial tissue in rheumatoid joint is considered to be the important cause of joint destruction (1). The osteoarthritic synovium does not participate in the degradative process as actively as the rheumatoid synovium. Nevertheless, low-grade chronic inflammation of the synovial membrane and infiltration of mononuclear leucocytes are associated with the osteoarthritic condition (2). Macrophages play an important role in the chronic inflammatory processes, owing to their phagocytic activity, secretory function, and participation in the immunological events (3). These cells also secrete the enzymes which degrade cartilage matrix (4-6). Most attempts to detect significant amounts of degradative enzymes in normal cartilage have not been successful, although low levels of cathepsins and some metallo-

0006-291X/78/0851-0490$01.00/0

proteases have been detected recently (2,7). Cathepsin D activity increases by about 2-3 fold in osteoarthritic cartilage (8), and this cartilage produces small amounts of collagenase after prolonged incubation in culture (9). It is therefore believed that a major part of cartilage degradation occurs via enzymes from extrinsic sources, rather than by those synthesized by chondrocytes. In this communication, we describe the effect of macrophages on articular chondrocytes in culture and report that macrophages release some factor(s) which activates the synthesis and secretion of collagenase and neutral proteases by chondrocytes.

MATERIALS AND METHODS

Chondrocytes were isolated from the articular cartilage of knee and hip joints of rabbits by enzymic digestion (10) and plated at a density of 3×10^5 cells per 25 cm^2 flask. The cells were grown to confluency in monolayer cultures in Ham's F-12 nutrient mixture (GIBCO), containing fetal calf serum (10% v/v), antibiotics and in an atmosphere of 5% Co_2 in air (10).

Male rabbits (1.5 - 2 kg) were injected intraperitoneally with 10 ml of sterile light mineral oil to induce cellular infiltration. After 96 hr, the peritoneum was washed with Dulbecco's Modified Eagle's Medium (DMEM), supplemented with heparin (2 U/ml), 2 mM glutamine, 10% fetal calf serum and antibiotics. The cells were collected by centrifugation and plated in the same medium (without heparin) at a density of 1×10^6 cells per cm^2. After incubation for 4-6 hr in an atmosphere of 5% CO_2 in air, non-adherent cells were removed and the remaining cells were washed 2-3 times with the serum-free medium. At least 95% of the cells were macrophages, as judged by their morphology and their ability to phagocytize latex particles. These cells were activated by incubation overnight in the serum-free medium containing lipopolysaccharides (LPS; 30 µg/ml) from Escherichia coli (055:B5, Difco Laboratories) (4). In one experiment the cells received no LPS. The next day, the medium was collected and centrifuged to remove any floating cells.

The confluent chondrocytes ($1.8 - 2 \times 10^6$ cells per flask) were incubated with macrophage-conditioned medium (MCM), diluted 1:4 with DMEM containg serum, for various time periods. The conditioned medium was removed and fresh DMEM with serum was added. This medium was changed daily for 4 days. All the media were dialyzed against 50 mM Tris-HCl buffer, pH 7.5, containing 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Na azide, and stored frozen. Prior to the enzyme analyses, 200 µl aliquots of the media were treated with 50 µg trypsin

(Worthington TRTPCK) for 10 min at 25°, followed by 150 µg soybean trypsin inhibitor (Worthington SI) for 20 min at 25°. This procedure converted the latent forms of enzymes to active enzymes (11,12).

Collagenase activity in the chondrocyte medium was assayed by incubation with reconstituted fibrils of 200 µg of [^{14}C] glycine-labelled rat-skin collagen (sp. act. 50,000 dpm/mg) (13) for 2 hr at 37°. All assays included a sample incubated with trypsin (0.01%), which served as a control for collagen denaturation. One unit of collagenase activity represents the lysis of 1 µg of reconstituted collagen per min at 37°C.

The assay for activity of neutral proteases was carried out in two ways. (A) [^{14}C] leucine-labelled globin was prepared from the reticulocytes of phenylhydrazine-treated rabbits (14). [^{14}C] globin (200 µg, sp. act. 17,000 dpm/mg) was incubated at pH 7.5 with the medium for 2 hr at 37°. Cold trichloracetic acid (10% v/v) was added to precipitate the undegraded globin, and the radioactivity released in the supernatant was estimated. One unit of neutral protease activity is defined as 1 µg of globin degraded per min at 37°. (B) Bovine articular cartilage (5 g) was incubated with Ham's F-11 Nutrient mixture (10 ml), containing 50 µC of [^{14}C] glycine (sp. act. 100 mCi/mM), for 6 hr at 37° C. [^{14}C] proteoglycans were isolated by extraction of cartilage with 4 M guanidinium chloride and CsCl density gradient centrifugation (15). The subunits (PGS) were further purified by chromatography with Sepharose 2B and Sephadex G-200 columns. [^{14}C] PGS (100 µg; sp. act. 16,000 dpm/mg) was incubated with the chondrocyte medium in 0.2 M PO_4 buffer, pH 7, at 37° C for 16 hr. Cetylpyridinium chloride (5%) was added to complex with PGS. Cold trichloroacetic acid (9%) was then added and the mixture centrifuged (16). The degradation of PGS was measured by counting the radioactivity or estimating uronic acid content of the supernatant (17). One unit of proteoglycanase activity represents the degradation of 1 µg PGS per hr at 37° C.

RESULTS AND DISCUSSION

The conditioned medium of LPS-treated (MCM) macrophages did not contain detectable amounts of collagenase or neutral proteases. Nevertheless, when added to the confluent chondrocytes, it induced the synthesis of enzymes by these cells. In the preliminary experiments, chondrocytes were treated with MCM for various time periods and the extent of induction of enzyme secretion was measured. Incubation for 24 hr with MCM led to the maximum enzyme production. Also, the optimum stimulatory effect was observed when MCM was diluted 1:4 (Table 1).

TABLE I

Stimulatory Effect of Various Concentrations of MCM on the Enzyme-Synthesis by Chondrocytes

Dilution	Collagenase Units*	Neutral Proetease (Globin) Units*
None	7.06 ± 0.57	27.00 ± 1.04
1:2	8.00 ± 0.23	30.75 ± 0.30
1:4	8.06 ± 0.56	30.98 ± 0.99
1:10	5.38 ± 0.38	20.27 ± 0.84

*Each value represents (mean ± S.D.; n=4) the cumulative amounts of enzyme released in the medium in 48 hr.

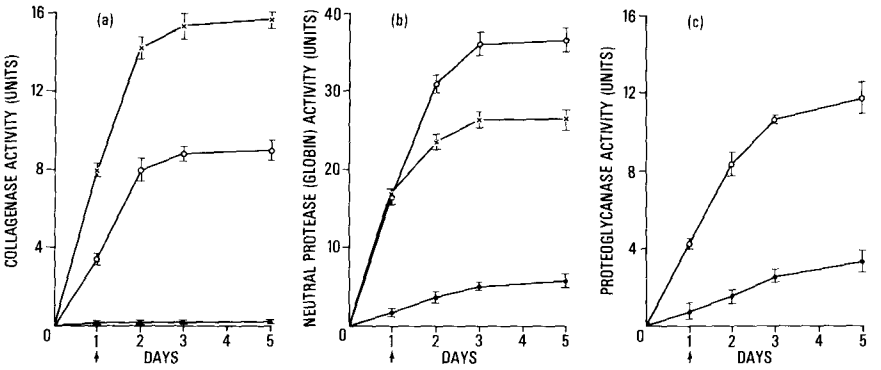

Fig. 1. Secretion of enzymes by chondrocytes as a result of treatment with MCM. Each point represents mean ± S.D. (n = 4) of culmulative total of the enzyme released in 4 ml medium of each flask (1.8 - 2.0 x 10^6 cells). One group of cells was not exposed to MCM (----), while the other was treated with MCM, diluted 4 fold with DMEM, containing serum (O---O), or without serum (X----X). The arrow indicates the point of termination of MCM treatment, after which the cells received DMEM, with or without serum daily. (a) collagenase; (b) nonspecific neutral protease; and (c) proteoglycanase activity at pH 7.

The chondrocytes produced significant amounts of collagenase and neutral proteases during incubation with MCM and on the following day (Fig. 1 a-c). The nonspecific neutral protease activity was many folds higher than proteoglycanase activity. In the absence of serum in medium, the total collagenase production by the cells

increased two fold, while the nonspecific neutral protease activity was slightly reduced. The increase in magnitude of proteoglycanase activity over the control cell population was the same in the presence or absence of serum in the medium. The collagenase activity was completely inhibited by 10 mM EDTA and 5% fetal calf serum; 70% inhibition was seen with 2 mM dithiothreitol, while soybean trypsin inhibitor had no effect. Likewise, neutral protease activity was totally inhibited by EDTA and dithiothreitol. 70% inhibition occurred with 5% serum and no inhibition with the soybean trypsin inhibitor.

The stimulatory factor in MCM was not destroyed by heat (56^{o}, 30 min), or by repeated freezing and thawing, and was stable at -20^{o} for at least a month. The stimulating activity was lost significantly by dialysis of MCM in dialysis tubing with molecular weight cut-off of 12,000. The conditioned medium from macrophages receiving no LPS showed minimal effect on chondrocytes (Table 2).

Several reports indicate that macrophages obtained from different sources such as mouse or guinea pig peritoneum, rabbit bone marrow, alveoli, etc. (4-6,18) secrete collagenase and neutral proteases in the medium. In most of these cases, activation of the cells with LPS, thioglycolate, or latex particles is necessary for enzyme synthesis. In the present studies, rabbit peritoneal macrophages did not secrete these enzymes with or without LPS treatment. However, the LPS-treated macrophages produced some mediator(s) which stimulated enzyme synthesis by chondrocytes. Similar instances have been reported where one cell population or its products activate certain functions of other cell types. For example, guinea pig peritoneal macrophages secrete collagenase in culture when treated with the medium of concanavalin A or endotoxin-stimulated lympho-

TABLE II

Effect of Various Treatments of MCM on its Capability to Induce Enzyme-Secretion by Chondrocytes

Treatment	Collagenase Units*	Neutral Protease (Globin) Units*	Proteoglycanase Units*
No LPS	0.50 ± 0.12	7.11 ± 0.71	3.00 ± 0.75
Activated with LPS	8.06 ± 0.56	30.98 ± 1.05	8.31 ± 0.65
Heat Treatment (56 ; 30min)**	7.81 ± 0.85	28.99 ± 3.04	9.11 ± 0.80
Freezing and Thawing	7.92 ± 0.26	29.37 ± 0.85	7.99 ± 0.70
Storage at -20 for 1 month**	8.82 ± 0.43	30.42 ± 1.90	8.85 ± 0.68
Dialysis**	0.03 ± 0.01	2.44 ± 0.77	3.49 ± 0.79

*The values represent (mean ± S.D.; n=4) the cumulative enzyme activity in 48 hr.

**All the treatments were given to MCM from LPS-activated macrophages.

cytes (19); a factor from human peripheral blood lymphocytes enhances the collagenase production by rheumatoid synovial cells (20).

The finding that the products of macrophages can turn on enzyme synthesis by cartilage cells may be of physiological importance, since chronic inflammatory conditions are associated with an active influx of macrophages. In the presence of macrophages *in vivo*, chondrocytes may secrete significant amounts of collagenase and neutral proteases spontaneously, and participate in the cartilage destruction process very actively.

REFERENCES

1. Harris, E. D., Jr. and Krane, S. M. (1974) N. Eng. J. Med. 291, 557-563, 605-609.
2. Howell, D. S., Sapolsky, A. I., Pita, J. C. and Woessner, J. F. (1976) Seminars in Arthr. Rheum. 5, 365-383.
3. Davies, P. and Allison, A. C. (1972) Immunobiology of Macrophages, pp. 427-461, Academic Press, New York.
4. Werb, Z. and Gordon, S. (1975) J. Exp. Med. 142, 346-360.

5. Wahl, L. M., Wahl, S. M., Mergenhagen, S. E. and Martin, G. R. (1974) Proc. Nat. Acad. Sci. (USA) 71, 3598-3601.
6. Hauser, P. and Vaes, G. (1978) Biochem. J. 172, 275-284.
7. Sapolsky, A. I., Keiser, H., Howell, D. S. and Woessner, J. F. (1976) J. Clin. Invest. 1030-1041.
8. Sapolsky, A. I., Altman, R. D., Woessner, J. F. and Howell, D. S. (1973) J. Clin. Invest. 52, 624-633.
9. Ehrlich, M. G., Mankin, H. J., Jones, H., Wright, R., Crispen, C. and Vigliani, G. (1977) J. Clin. Invest. 226-233.
10. Deshmukh, K. and Kline, W. G. (1976) Eur. J. Biochem. 69, 209-215.
11. Dayer, J. M., Krane, S. M., Russell, G. G. and Robinson, D. R. (1976) Proc. Nat. Acad. Sci. (USA) 73, 945-949.
12. Vaes, G., Eekhout, Y., Linaers-Claeys, G., Francois-Gillet, C. and Druetz, J. C. (1978). Biochem. J. 172, 261-274.
13. Nagai, Y., Lapiere, C. M. and Gross, J. (1966). Biochemistry 5, 3123-3130.
14. Gilbert, J. M. and Anderson, W. F. (1970) J. Biol. Chem. 245, 2342-2349.
15. Hascall, V. C. and Sajdera, S. W. (1970) J. Biol. Chem. 245, 4920-4930.
16. Sapolsky, A. I., Woessner, J. F. and Howell, D. S. (1975) Anal. Biochem. 67, 649-654.
17. Bitter, T. and Muir, H. (1962) Anal. Biochem. 4, 330-334.
18. Werb, Z. (1978) J. Exp. Med. 147, 1695-1712.
19. Wahl, L. M., Wahl, S. M., Mergenhagen, S. E. and Martin, G. R. (1975) Science 187, 261-263.
20. Dayer, J. M., Russell, R. G. G. and Krane, S. M. (1977) Science 195, 181-183.