Vol. 86, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1979 Pages 71-77

ACTIVATED MACROPHAGES DIGEST THE EXTRACELLULAR MATRIX PROTEINS PRODUCED BY CULTURED CELLS

Peter A. Jones[1] and Timothy Scott-Burden[2]

MRC Unit for Molecular and Cellular Cardiology
Box 63, Tygerberg 7505, South Africa

Received November 15,1978

SUMMARY: Activated mouse peritoneal macrophages were cultured directly on the extracellular matrix proteins produced by smooth muscle cells in vitro. The breakdown of the connective tissue proteins to the level of amino acids was followed by observing the release of radioactivity from matrices labelled with [^{3}H]proline. These studies showed that macrophages produce enzymes capable of digesting the matrix and indicated a major role for the macrophage plasminogen activator in this digestion.

The extracellular matrix is a complicated mixture of insoluble structural proteins, proteoglycans, soluble proteins and diffusible metabolites. Its breakdown is of fundamental importance to many normal and pathological conditions such as tissue remodelling, wound healing and tumor invasion. Matrix breakdown probably requires a battery of enzymes working together, since many of the components, eg. collagen and elastin, are only efficiently degraded by specific proteases (1,2).

In this communication, we describe the complete degradation, by activated mouse peritoneal macrophages, of the matrix proteins synthesized by cultured smooth muscle cells. The smooth muscle cells cultured in the presence of [^{3}H]proline, synthesize an extensive radioactively labelled matrix, containing glycoprotein(s), elastin and collagen,which remains on the bottom of culture dishes following removal of the producer cells by mild alkaline treatment (3). Activated macrophages which secrete plasminogen activator (4), elastase (5) and collagenase (6) were then cultured on the matrix so that its breakdown could be followed.

Present Addresses: [1]Division of Hematology-Oncology, Childrens Hospital of Los Angeles, 4650 Sunset Boulevard, Los Angeles, California 90027. [2]Department of Medical Biochemistry,University of Cape Town, Observatory, Cape Town 7925.

0006-291X/79/010071-07$01.00/0

MATERIALS AND METHODS

Production of Dishes Containing Labelled Matrix: Smooth muscle cells obtained from 4th to 8th passage stock cultures (3) were seeded into 35mm dishes (10^5/dish). The cultures were treated with 50 µg/ml of ascorbate on the following day and received fresh ascorbate daily. [^{3}H]proline (1 µC/ml) was added 5 days after seeding and the matrix prepared 7 days later. The cultures were washed once with phosphate buffered saline and the cells lysed in 0.25N NH_4OH for 30 min at room temperature. The matrix was then vigorously washed with distilled water followed by 70% ethanol and allowed to dry at 37°C. The dried dishes were allowed to stand for 5 min in 70% ethanol and then washed three times with sterile saline before the addition of cells in experiments where sterility was required. Sequential enzyme analysis of these matrices demonstrated that 34% of the proline radioactivity was released by trypsin, a further 43% by elastase and 21% by collagenase.

Preparation of Activated Macrophages: Thioglycollate-activated mouse peritoneal macrophages were prepared as described by Gordon et al. (7) and cultured in McCoy's 5A medium. Acid-treated serum and plasminogen depleted serum were prepared by standard methods (8).

Digestion Studies: Dishes containing [^{3}H]proline-labelled matrix were incubated at 37°C under the conditions outlined in the text. Samples (100 µl) were withdrawn at the end of the required incubation times, added to 5 ml of Instagel (Packard Instruments, Downers Grove, Illinois) and the radioactivity determined in a Packard Liquid Scintillation Spectrometer. The dishes were then washed and the residual radioactivity determined following overnight digestion in 2N NaOH at 37°C and neutralization with concentrated HCl (3). The total radioactivity present on each dish was then determined by the addition of the values obtained for the residual and solubilized radioactivity and the results expressed in each case as percentages of the total radioactivity released.

Sephadex Chromatography: Analyses of digestion products were carried out on a Sephadex G-25 column (26 x 1.8 cm). The running buffer was 0.2M Tris HCl pH 7.6, containing 0.1% sodium azide and 0.1 mg/ml bovine serum albumin. Fractions of 1.5 ml each were collected and the radioactivity present in 0.5 ml samples determined in 5 ml of Instagel. The column was calibrated with Blue Dextran which was recovered in fraction 14 and with [^{3}H]proline, which was recovered in fraction 27. Recovery of radioactivity from the columns was consistently found to be of the order of 95%.

RESULTS

Matrix Degradation by Living Cells. The kinetics of degradation of the matrix by living macrophages plated directly onto the labelled proteins are shown in Fig. 1. The rate of digestion was dependent on the serum supplement, with the slowest degradation occurring under serum-free conditions and the most rapid in the presence of dog serum which contains high levels of plasminogen (9). Destruction of the protease inhibitors in fetal bovine serum by acid treatment, caused an increase in the hydrolysis rate.

The marked effects of different serum supplements on the rate of hydrolysis suggested that plasminogen might play some part in the digestion of

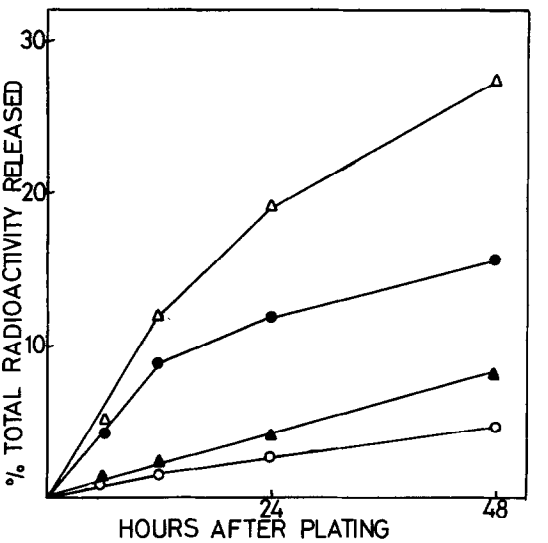

Figure 1. The effect of serum supplement on the digestion of [^{3}H]proline-labelled matrix by living macrophages. Activated macrophages (10^6) were seeded into 35mm dishes containing approximately 10^5 cpm/dish in medium containing no serum (0); 10% fetal bovine serum (▲); 10% acid-treated fetal bovine serum (●); or 10% dog serum (Δ). Aliquots were removed where indicated for radioactivity determinations and the values obtained for dishes containing no added macrophages were subtracted from the results shown.

the matrix, and this possibility was explored in the experiments shown in Table 1. The presence of plasminogen caused a considerable stimulation of hydrolysis, the most rapid solubilization occurring under serum-free conditions with added plasminogen. Plasminogen depletion resulted in a large decrease in the hydrolysis rate and this could be significantly increased by re-addition of the serum zymogen. Other experiments (not shown) demonstrated that this level of plasminogen, which is of the same order of magnitude as that reported for undiluted human serum (100-200 μg/ml (10)), caused a 21% release of radioactivity from the matrix after 48 hr in the presence of urokinase. The experiments therefore show that some matrix components are hydrolysed by plasmin, and that the presence of plasminogen is necessary for the maximum hydrolysis rate.

Analysis of Digestion Products. The supernatant medium from dishes containing macrophages plated on [^{3}H]proline-labelled matrices was analyzed by

Table 1

Effect of Plasminogen on Matrix Digestion by Living Macrophages

Serum Supplement	Plasminogen added (Final concentration)	% Total Radioactivity solubilized in 48 hr
None	None	4.3
None	7.5 µg/ml	27.6
10% Acid-treated fetal bovine serum	None	15.4
10% Plasminogen depleted acid-treated fetal bovine serum	None	0.8
10% Plasminogen depleted acid-treated fetal bovine serum	7.5 µg/ml	8.5

The effect of plasminogen on the digestion of [^{3}H]proline-labelled matrix by living macrophages. Activated macrophages (10^6) were seeded into 35mm dishes containing approximately 2 x 10^5 cpm/dish in the indicated medium. The dishes were then incubated at 37°C and the percentage of the total radioactivity solubilized after 48 hr determined. Values for dishes containing no cells (approximately 1% release in 48 hr) have been subtracted from the values shown.

Sephadex G-25 gel filtration (Fig. 2). Most of the radioactive peptides released after 12 hr incubation were excluded from the column. After 24 hr a small amount of radioactivity was found to elute in the position where [^{3}H]proline eluted from the column (Fraction 27), and the percentage of radioactivity in this position increased linearly with time. The elution profiles also showed that no peptides were found with sizes intermediate between the excluded material and free amino acids.

DISCUSSION

The substrate which we have used in these experiments contains glycoprotein(s), elastin and collagen (3) and as such contains the main structural components of connective tissue (11). We have not as yet identified the glycoprotein component, but since it contains fucose and has an apparent molecular weight of 250,000 (3), it is likely to be fibronectin (12,13) and/or the microfibrillar protein of the elastic fiber (14).

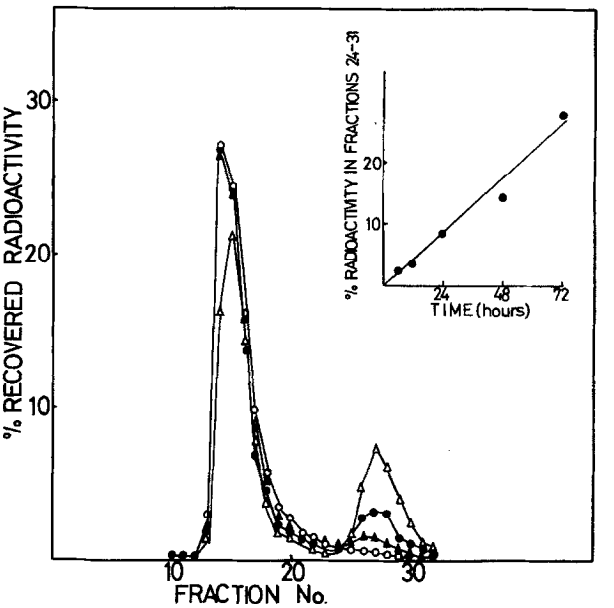

Figure 2. Sephadex G-25 column chromatography of material solubilized by activated macrophages. Activated macrophages (10^6) were seeded in medium with 10% acid-treated fetal bovine serum, into dishes containing [^{3}H]proline-labelled matrix (approximately 10^5 cpm/dish). Samples of the medium were analyzed on the column after 12 hr(0); 24 hr(▲); 48 hr(●); and 72 hr(Δ). Inset. Plot of percentage radioactivity recovered in fractions 24-31 at different times, versus incubation time.

The only effective procedure to remove the producer cells and prepare a suitable matrix, was the mild alkaline treatment described. EDTA or non-ionic detergents such as Triton X-100 were ineffective and although sodium dodecyl sulfate rapidly solubilized the cells, the detergent remained firmly bound to the matrix so that it was subsequently detrimental to the added macrophages. The drying step was introduced to allow for the production of large numbers of replicate samples and the specificity of all of the components to enzymatic digestion was well maintained (3).

The experiments with living macrophages grown in contact with the substrate, indicated a major physiological role for the plasminogen activator produced by these cells (4) in the digestion of the extracellular matrix. The plasmin generated may not only hydrolyse some of the substrate (eg. the glyco-

protein component), thus allowing the other enzymes access to their substrates, but may also be implicated in the activation of secreted zymogens such as procollagenase (15). Our studies therefore provide direct evidence for the importance of plasminogen activator in the breakdown of connective tissue proteins.

Activated macrophages were also found to complete the hydrolysis of the matrix proteins to small molecules. The identity of the radioactivity in the position where [^{3}H]proline eluted from the column was not determined, but was probably due to this amino acid as has been found for other labeled substrates (16). The results would be consistent with a model in which the secreted proteases hydrolyze the substrate and release peptides with a chain length greater than 20 amino acids (the approximate exclusion limit of Sephadex G-25). The peptides would then be internalized and further hydrolyzed by the macrophage lysosomal acid proteases to small molecules and released into the medium. Such a model would be in accordance with currently-held concepts of macrophage physiology (16).

Acknowledgements: This work was supported by research grants from the South African Medical Research Council and the National Cancer Association of South Africa. Professor W. Gevers is thanked for his help in discussions.

References

1. Harris, E.D., and Krane, S.M. (1974) New Eng. J. Med. 291, 557,605,652.
2. Hartley, B.S., and Shotton, D.M. (1971) The Enzymes, ed. by P.D. Boyer, Vol. 3, pp. 323-373, Academic Press, New York.
3. Jones, P.A., Scott-Burden, T., and Gevers, W. (1978) Proc. Natl. Acad. Sci. U.S.A. (in press).
4. Unkeless, J.C., Gordon, S., and Reich, E. (1974) J. Exptl. Med. 139, 834-850.
5. Werb, Z., and Gordon, S. (1975) J. Exptl. Med. 142, 361-377.
6. Werb, Z., and Gordon, S. (1975) J. Exptl. Med. 142, 346-360.
7. Gordon, S., Todd, J., and Cohn, Z.A. (1974) J. Exptl. Med. 139, 1228-1248.
8. Jones, P., Benedict, W., Strickland, S., and Reich, E. (1975) Cell 5, 323-329.
9. Laug, W.E., Jones, P.A., and Benedict, W.F. (1975) J. Natl. Cancer Inst. 54, 173-179.
10. Sherry, S. (1972) Hematology, ed. by W.J. Williams, E. Beutler, A.J. Ersler, and R.W. Rundles, New York: McGraw-Hill.
11. Rucker, R.B., and Tinker, D. (1977) Int. Rev. Exp. Pathology 17, 1-47.
12. Sear, C.H.J., Grant, M.E., and Jackson, D.S. (1977) Biochem. J. 168, 91-103.

13. Engvall, E., Ruoslahti, E., and Miller, E.J. (1978) J. Exptl. Med. 147, 1584-1595.
14. Muir, L.W., Bornstein, P., and Ross, R. (1976) Eur. J. Biochem. 64, 105-114.
15. Werb, Z., Mainardi, C.L., Vater, C.A., and Harris, E.D. (1977) New Eng. J. Med. 296, 1017-1023.
16. Cohn, Z.A. (1975) Proteases and Biological Control, ed. by E. Reich, D.B. Rifkin, and E. Shaw, pp. 483-493, Cold Spring Harbour Laboratory.