PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 173, 324–327 (1983)

Modulation of Plasminogen Activator Activity in Human Skin Fibroblasts Infected with Mycoplasmas (41650)

VICTOR B. HATCHER

Department of Biochemistry and Medicine, Albert Einstein College of Medicine, Montefiore Medical Center, 111 East 210th Street, Bronx, New York 10467

Abstract. Secreted and cell-associated plasminogen activator was measured in human skin fibroblasts infected with mycoplasmas. The infected fibroblasts exhibited significantly higher levels of secreted and cell-associated plasminogen activator activity compared to uninfected skin fibroblasts. Subconfluent infected fibroblasts had increased plasminogen activator activity compared to confluent infected fibroblasts. Plasminogen activator may be important in tissue destruction in disease states in which mycoplasma infection has been implicated.

Plasminogen activator, a member of a specific group of serine proteases capable of activating plasminogen to plasmin, is synthesized and released by a variety of cell types and elevated in several virally and chemically transformed cells in culture (1–3). The generated plasmin is capable of stimulating cell division (4), modifying cell surfaces (4, 5), enhancing cell migration (6), and activating specific proteases (7). In the present study, we have determined secreted and cell-associated plasminogen activator (PA)[1] activity in uninfected and mycoplasma-infected human skin fibroblasts. We observed that fibroblast cultures with increased PA activity corresponded to cultures contaminated with mycoplasmas detected during routine screening.

Materials and Methods. *Cells.* The human skin fibroblasts which were established from human foreskins were grown routinely in Dulbecco's modified Eagle's medium on 60-mm Falcon plates with 10% fetal calf serum in an atmosphere with 5% CO_2 at 37°C. Matched fibroblasts between the 4th and 12th passage were utilized in the experiments. Subconfluent cells, which were rapidly proliferating, were utilized 48 hr after the cells were subcultured. Confluent cells were used several days after no increase in the cell number was measured. Cell counts were performed on duplicate plates utilizing a hemocytometer. Cell viability was determined using Eosin Y dye exclusion. Mycoplasma screening was performed routinely by agar plate cultivation and fluorescent staining for cytoplasmic DNA by Dr. G. J. McGaritty, Institute for Medical Research, Camden, New Jersey (8). Immunofluorescent staining was used to identify the *Mycoplasma hyorhinis* antigen in all infected strains. The fibroblasts were naturally infected with the mycoplasmas. *Mycoplasma hyorhinis* was obtained from the American Type Culture Collection, Rockville, Maryland and cultured by Dr. Irna Weber, Department of Medicine. Urokinase was obtained from Sigma Chemical Company, St. Louis, Missouri.

Plasminogen activator activity. The subconfluent or confluent cells were washed three times with fresh serum-free medium (SFM). The fibroblasts were placed in SFM and incubated for either 48 or 72 hr at 37°C. Mycoplasmas were placed in SFM in culture dishes containing no fibroblasts and assayed under the same conditions. Mycoplasma organism in broth were also analyzed. In the measurement of secreted PA activity, aliquots of the conditioned medium were removed at 3, 24, 48, and 72 hr, acidified to pH 3.5 with 1 *N* HCl, to remove protease inhibitors (9), and assayed. In experiments with several cell lines, similar results were obtained when the medium was not acidified (results not shown). In the measurement of cell-associated PA activity, duplicate plates were incubated in SFM at 37°C for either 3, 24, or 48 hr. The cells were scraped off the plates with a rubber policeman and extracted by freezing and thawing (3×) in 1 ml of 0.05 *M* sodium phosphate buffer, pH 7.5, containing 1 *M* KCl and 0.1%

[1] Abbreviations: PA, plasminogen activator; SFM, serum-free medium.

0037-9727/83 $1.50

Triton X-100. Triplicate aliquots (50 μl) from either the conditioned medium or the homogenate were added to 20 μl of a 0.1 *M* Tris–HCl buffer, pH 8.1, containing 10 μg of purified human plasminogen and 0.2% Triton X-100 in microcentrifuge tubes. The human plasminogen which was purified from human plasma according to the method described by Deutch and Mertz (10) did not significantly degrade acetyl[^{3}H]casein. Following the addition of 20 μl acetyl[^{3}H]casein (9.6 μg, 200,000 cpm) prepared as previously described (11) the samples were vortexed and incubated at 37°C for 2 hr.

Plasminogen-independent proteolysis was measured by incubating conditioned medium (50 μl) with 20 μl of a 0.1 *M* Tris–HCl buffer, pH 8.1, containing 0.2% Triton X-100 and 20 μl of acetyl[^{3}H]casein (9.6 μg, 200,000 cpm) at 37°C for 2 hr. The reactions were terminated and TCA soluble peptides counted as previously described (11). Minimal amounts of plasminogen-independent proteolysis was observed in the conditioned medium or homogenates from the infected or uninfected cells. Plasminogen activator activity was determined by substrating the results from the plasminogen-independent proteolysis from the results from the plasminogen-dependent proteolysis. Benzamidine inhibition was measured by preincubating the samples (50 μl) with 0.1 *M* Tris–HCl buffer (20 μl), pH 8.1, containing 0.2% Triton X-100 and 13 m*M* benzamidine for 10 min at 4°C. The assay was then performed as described. One unit of PA activity was defined as the amount of enzyme which solubilized 1,000 cpm of acetyl[^{3}H]casein. In a standard urokinase assay, 0.005 Plough units solubilized 5000 cpm of acetyl[^{3}H]casein under our experimental conditions.

Results and Discussion. Plasminogen activator activity was assayed in the conditioned medium from seven strains of subconfluent human skin fibroblasts infected with mycoplasmas and nine different uninfected human skin fibroblast strains. The cumulative results from several experiments are shown in Fig. 1. The uninfected skin fibroblasts exhibited low levels of PA activity up to 72 hr. The time course of accumulation of PA in the culture medium from skin fibroblasts infected with mycoplasmas indicated that the infected cells

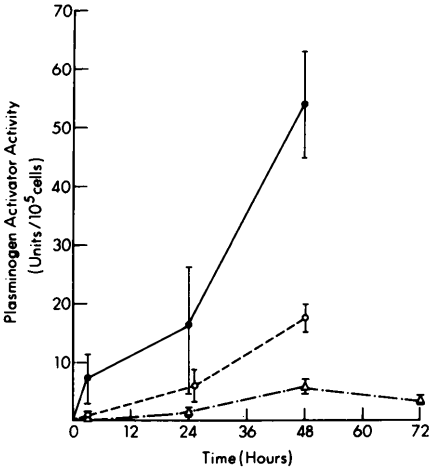

FIG. 1. Secreted plasminogen activator activity in conditioned medium from mycoplasma infected and uninfected subconfluent human skin fibroblasts. Plasminogen activator activity in conditioned medium from seven strains of mycoplasma-infected human skin fibroblasts (●). Plasminogen activator activity in conditioned medium treated with benzamidine from seven strains of mycoplasma-infected human skin fibroblasts (○). Plasminogen activator activity in conditioned medium from nine strains of uninfected human skin fibroblasts (△). Each strain was analyzed individually and the cumulative results presented as mean ± standard deviation.

accumulated increased amounts of measurable PA in the medium compared to the uninfected cells. The PA in the conditioned medium from the infected cells were partly inhibited by benzamidine. The mycoplasmas did not affect the cell counts or viability of the skin fibroblasts. The contaminating microorganism in the skin fibroblasts with high PA activity was identified as *M. hyorhinis* by the Institute for Medical Research, Camden, New Jersey. The cumulative results of cell-associated PA activity in the homogenates are shown in Fig. 2. No difference in cell-associated PA activity was observed between the uninfected and infected cells incubated in SFM for 3 or 24 hr. The homogenates from the infected fibroblasts incubated in SFM for 48 hr had elevated cell-associated PA activity compared to the uninfected fibroblasts. Secreted and cell-associated PA activity from confluent and subconfluent mycoplasma-infected cells are shown in Table I. Conditioned medium (48 hr) from subconfluent cells contained higher PA activity compared to con-

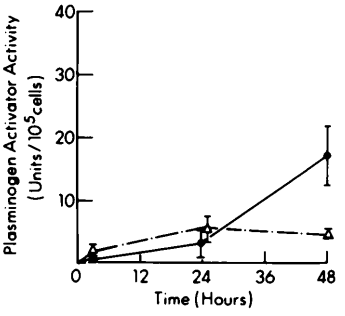

FIG. 2. Cell-associated plasminogen activator activity in extracts from mycoplasma-infected and uninfected subconfluent human skin fibroblasts. Plasminogen activator activity in extracts from seven strains of mycoplasma-infected human skin fibroblasts (●). Plasminogen activator activity in extracts from nine strains of uninfected human skin fibroblasts (△). Each strain was analyzed individually and the cumulative results presented as mean ± standard deviation.

ditioned medium from confluent cells. Higher cell-associated PA activity was also observed in homogenates from the subconfluent cells compared to homogenates from the confluent cells. Similar results were obtained when medium was changed each day and PA determined. Mycoplasma organisms in broth or in tissue culture dishes did not contain or secrete any PA activity at any time period.

Increased cellular and secreted PA activity was observed in mycoplasma-infected human skin fibroblasts. In the mycoplasma-infected fibroblasts, the source of the PA is not from the mycoplasma. Although plasminogen activator activity has not been extensively investigated in mycoplasmas, this study demonstrates that *M. hyorhinis* does not exhibit PA activity under our experimental conditions. Aminopeptidase activity has been demonstrated in several strains of mycoplasmas (12). Proteolytic activity has been demonstrated with a synthetic tetrapeptide substrate S-2222 in extracts of *M. pneumonia* (13).

Mycoplasmas have been implicated in the establishment of connective tissue disease in certain animal species (14, 15). Kluve *et al.* (16) have shown that mycoplasmas induce inactive collagenase in Balb/c 3T3 cells. Increased collagenase activity have been implicated in connective tissue destruction (17, 18). Plasmin generated by PA can activate collagenase (7). Plasminogen activator activity in fibroblasts infected with mycoplasmas may be important in the development and persistance of tissue destruction.

TABLE I. PLASMINOGEN ACTIVATOR ACTIVITY IN SUBCONFLUENT AND CONFLUENT FIBROBLASTS INFECTED WITH MYCOPLASMAS[a]

	Plasminogen activator activity (Units/10^5 cells)	
Condition	Secreted	Cell associated
Subconfluent	55.6 ± 14.3	13.5 ± 5.6
Confluent	25.5 ± 22.2	5.9 ± 3.3

[a] Subconfluent and confluent mycoplasma-infected skin fibroblasts (three strains) were incubated in SFM for 48 hr. The 48-hr conditioned medium was removed and assayed for PA activity. The cells were then extracted and assayed for cell associated PA activity as described in Materials and Methods. Mean ± standard deviation from two different experiments. One unit of PA activity was defined as the amount of enzyme which solubilized 1000 cpm of acetyl[^{3}H]casein.

This research was supported in part by grants from the Cystic Fibrosis Foundation, the National Institute of Health (AG 01732 and HL 16387), and the New York and American Heart Associations. Victor B. Hatcher is a recipient of the Ann Weinberg Cystic Fibrosis Research Scholarship Award, and an Established Fellow of the New York Heart Association. Dr. G. J. McGaritty, of the Institute for Medical Research, Camden, New Jersey, is acknowledged for performing the mycoplasma screening. We thank Eric Avezzano and Bobby Pink, Tufts University students, for assistance during the summer. Leslie Leibowitz is gratefully acknowledged for assistance in preparing the manuscript.

1. Rifkin DB, Loeb JN, Moore G, Reich E. Properties of plasminogen activators formed by neoplastic human cell cultures. J Exp Med **139**:1317–1328, 1974.
2. Laug WE, Jones PA, Benedict WF. Relationship between fibrinolysis of cultured cells and malignancy. J Nat Cancer Inst **54**:173–179, 1975.
3. Quigley JP. Proteolytic enzymes of normal and malignant cells. In: Hynes RO, ed. Surfaces of Normal and Malignant Cells. Sussex, England, Wiley, p247, 1979.
4. Blumberg PM, Robbins PW. Effect of proteases on activation of resting chick embryo fibroblasts and on cell-surface proteins. Cell **6**:137–147, 1975.
5. Hynes RO. Alteration of cell surface proteins by viral transformation and by proteolysis. Proc Nat Acad Sci USA **11**:3170–3174, 1973.
6. Ossowski L, Quigley JP, Reich E. Plasminogen, a

necessary factor for cell migration in vitro. In: Reich E, Rifkin D, Shaw E, eds. Proteases and Biological Control. Cold Spring Harbor, New York, Cold Spring Harbor Lab., p901, 1975.

7. Werb Z, Mainardi CL, Vater CA, Harris ED Jr. Activation of collagenase secreted by rheumatoid synovial cells—Evidence for a role of plasminogen activator. N Engl J Med **296**:1017–1023, 1977.
8. McGaritty GJ. Spread and control of mycoplasmal infection of cell-cultures In Vitro **12**:643–647, 1977.
9. Goldfarb RH, Quigley JP. Synergistic effect of tumor-virus transformation and tumor promoter treatment on production of plasminogen activator by chick embryo fibroblast. Cancer Res **38**:4601–4609, 1978.
10. Deutch DG, Mertz ET. Plasminogen: purification from human plasma by affinity chromatography. Science **170**:1095–1097, 1970.
11. Hatcher VB, Oberman MS, Lazarus GS, Grayzel AI. A cytotoxic proteinase isolated from human lymphocytes. J Immunol **120**:665–670, 1978.
12. Neill SD, Ball HJ. Aminopeptidase activity of *Acholeplasma-laidlawii, Mycoplasma-bovirhinis, Mycoplasma-dispar* and *Mycoplasma-bovis*. J Gen Microbiol **119**:103–107, 1980.
13. Chandler DK, Barile MF. Ciliostatic, hemagglutinating and proteolytic activities in a cell extract of *Mycoplasma-pneumoniae*. Infect Immun **29**:1111–1116, 1980.
14. Cassel GH, Cole BC. Mycoplasmas as agents of human disease. N Engl J Med **304**:80–89, 1981.
15. Washburn LR, Cole BC, Gelman MI, Ward JR. Chronic arthritis of rabbits induced by mycoplasmas. 1. Clinical, microbiologic and histologic features. Arthritis Rheum **23**:825–836, 1980.
16. Kluve B, Merrick WC, Stanbridge EG, Gershman H. Mycoplasmas induced collagenase in Balb/c 3T3 cells. Nature (London) **292**:855–857, 1981.
17. Dayer JM, Krane SM, Russell GG, Robinson DR. Production of collagenase and prostaglandins by isolated adherent rheumatoid synovial cells. Proc Nat Acad Sci USA **73**:945–949, 1976.
18. Erlich MG, Houle PA, Vigliani G, Mankin HJ. Correlation between articular cartilage collagenase activity and osteoarthritis. Arthritis Rheum **21**:761–765, 1978.

Received June 25, 1982. P.S.E.B.M. 1983, Vol. 173.