CELL SURFACE-ASSOCIATED AND RELEASED PROTEOLYTIC ACTIVITIES OF BOVINE AORTA ENDOTHELIAL CELLS

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Received August 9, 1976

Summary: We have demonstrated cell surface-associated and released proteolytic activity on bovine aorta endothelial cells, representing normal cells with regulated invasive properties. To demonstrate these proteolytic activities on viable cells grown in monolayer cultures, a new method was developed. The method consists of rolling modified plastic beads carrying covalently-linked [^{125}I]-labeled casein in contact with the cell surfaces or adjacent to the cell monolayers without actual contact. The rate of radioactive peptide release is proportional to the proteolytic activity. Released proteases are detected when no contact occurs between the substrate and the cells. When the bead-substrate complex is rolled over the surface of endothelial cells, a significant increase in released labeled peptides is observed which represents a cell surface-associated proteolytic activity. These activities may be relevant to the endothelial cell's invasive capacity and appear to be similar to the neutral proteolytic activity of transformed cells.

INTRODUCTION

Greatly increased fibrinolytic activity has been demonstrated with cells from primary cultures of spontaneous, virus and carcinogen-induced tumors (1,2). These transformed cells produce a plasminogen-activating neutral-protease, which leads to the increased fibrinolytic activity. Immunological identity between urokinase and the plasminogen activator produced by human ovarian carcinoma in tissue culture was recently reported (3). Although a correlation has been reported between the abilities of the cells to induce

*Supported by NCI Grant No. CA-14089 and by the California Division of the American Cancer Society Special Grant No. 744. Address: Research Building One, Room 245, 1720 Zonal Avenue, Los Angeles, CA 90033.

+Supported by NIDR Grant No. DE-0474.

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fibrinolysis, to grow in soft agar and to form tumors in immunosuppressed hosts (4), an active fibrinolytic system is a necessary but not a sufficient requirement for morphological changes accompanying transformation (5). Elevated levels of certain proteases and glycosidases were found in human malignant neoplastic tissues from highly invasive tumors of the breast and colon (6). These observations lead to the supposition that the increase in degradative enzyme activity may result in the release of tumor cells from contact inhibition and permit the cells to penetrate and to promote the dissolution of basement membranes.

Endothelial cells of blood vessels demonstrate the capacity of invading a tissue when an appropriate stimulus is provided; this stimulus could come from new tissue formation during development, wound healing and neoplastic growth. Although the mechanisms of invasion of one tissue by another are unknown, it is reasonable to postulate that during invasion an enzymatic component is responsible for the degradation of the invaded tissue's matrix. Since this matrix is composed mainly of proteins, proteases would seem to be a necessary component of the invasive process. From previous studies on cartilage, an avascular tissue containing protease inhibitors (7), and factors which inhibit the proliferation of endothelial cells of vascular origin (8), we have postulated the involvement of proteolysis in the vascularization of normal and neoplastic tissues (9). To further explore this hypothesis, we developed a new method which distinguishes between secreted and surface-associated neutral-protease activity. In this study we report the application of this method to bovine aorta endothelial cells capable of controlled tissue invasion.

MATERIALS AND METHODS

Cell Cultures. Primary calf aorta endothelial cells were obtained according to the method of Eisenstein et al. (7). Cells, identified as endothelial by the presence of Weibel-Palade bodies, were grown and subcultured in RPMI 1640 medium supplemented with 10 - 20% fetal calf serum and 20 mM Hepes (K. C. Biologicals, Kansas City, KS and Flow Laboratories, Rockville, MD). Thirty-five-mm plastic culture dishes (Falcon Plastics,

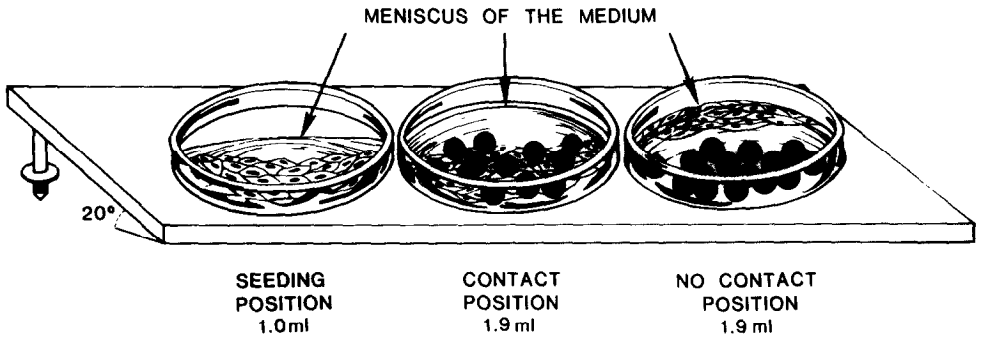

Figure 1. Diagrammatic illustration of the beads assay. Not drawn to scale. Cells are seeded only on the lower half of the dish. When the bead-substrate complex is introduced directly over the cell surface, "contact" occurs. In the "no contact" position, the dishes are turned, the cells are on the upper portion of the slope, and the bead-substrate complex is introduced in the lower section only.

Oxnard, CA) were seeded with 3 - 5 X 10^4 cells/ml over one-half of the petri dish surface (Figure 1). Culture dishes were on a tilted platform, so that the media covered only half the bottom area. Growth media, containing gentamycin and amphotericin B, was changed every second day until a density of 4 - 5 X 10^5 cells per dish was reached. Cell cultures from the second, fourth and sixth passages were used for the protease assay.

Solid-phase Protease Substrate. Casein, neutral protease-free (Bio-Bifunctionals, P. O. Box 54591, Los Angeles, CA) was [^{125}I]-iodinated and covalently coupled to modified latex beads (Bio-Bifunctionals), average diameter 26 μ, as previously described (10). The beads were extensively washed with 5 M Guanidine-HCl and resuspended in serum-free culture medium to contain 1 - 2 X 10^6 beads per ml of suspension.

Protease Assay. The assay was performed in the culture dishes, maintained on an adjustable tilted platform (Bio-Bifunctionals) as illustrated in Figure 1. The medium was aspirated and replaced with 1.8 ml of fresh medium with or without serum. Three sets of cultures were set up in quadruplicate. One-tenth ml of bead-suspension, carrying covalently-linked [^{125}I]-casein (3 - 5 X 10^5 cpm per 2 X 10^5 beads) was introduced to each culture. The first set, designated as "control", consisted of previously-washed petri dishes and medium only. In the second set, the dishes were turned so that the cells were on the upper slope and the bead-suspension was introduced to the lower half of the dish. In this configuration the solid-phase substrate was incubated in the same media without any chance of actual contact with cells, hence designated as "no contact" configuration. In the third set of cultures, the cells remained in the lower half of the dish and the bead-substrate complex was introduced directly over the viable cell surfaces, therefore designated as "contact" configuration. Incubations were carried out in a Hotpack CO_2 incubator. The media was sampled at various time intervals for the amount of [^{125}I]-label released from the solid-phase. At each time interval, 0.1 ml of the medium was removed and mixed with 1.0 ml of 1% bovine serum albumin in saline at 0°C. The mixture was centrifuged for 3 min at 500 g to settle any beads which may have been removed and 0.5 ml of the supernatant was tested for released radioactivity in a Packard 520 Autogamma Counter.

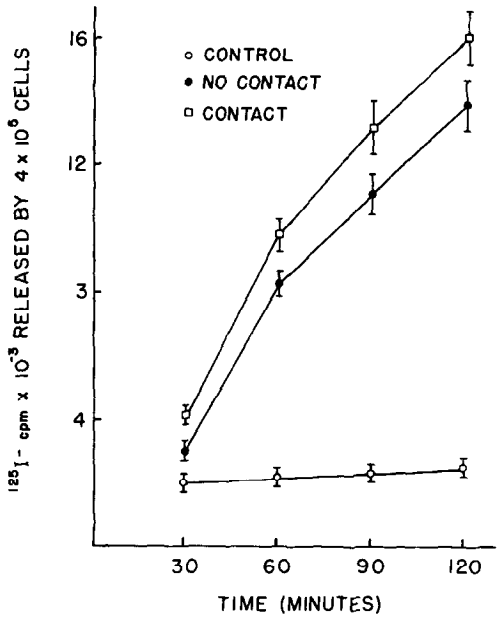

Figure 2. The release of radioactive peptides from the beads by bovine aorta endothelial cells. Each value represents the average of four determinations; the vertical bars represent standard deviations.

RESULTS AND DISCUSSION

The method described here allows one to quantitate the amount of proteolytic cleavage from a substrate covalently localized to latex bead surfaces. The assay is carried out in situ in the same culture dishes in which the cells were grown. There is no change in viability during the assay; the cells can be fully recovered and maintained in culture.

Spontaneous release from the beads alone (control samples) is usually less than 1% of the total radioactivity available on the beads (Figure 2). When the bead-substrate complex is presented in the "no contact" configuration, the cleavage of [^{125}I]-substrate is due to enzymes released by the cells. Since not every cleavage of a peptide bond necessarily results in the solubilization of a labeled fragment, it is difficult to quantitate the absolute enzyme levels. The majority of the enzymatic activity is of cellular origin, since endothelial cells maintained in 5% protease-free bovine serum albumin also release proteases.

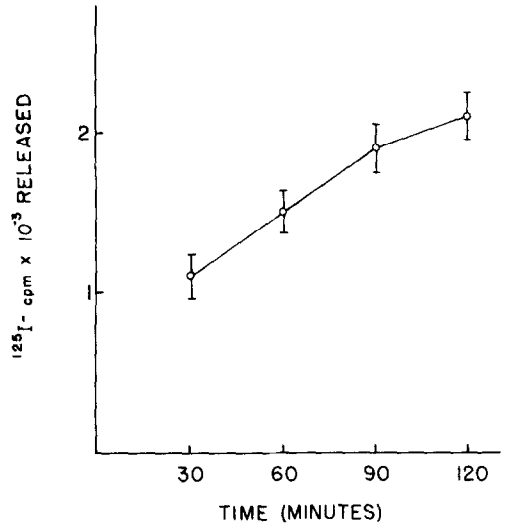

Figure 3. Cell surface-associated proteolytic activity due to contact between substrate and cell. Calculated from Figure 2.

Significantly greater proteolysis was observed when the substrate-bead complex is presented in contact with the cell surfaces. Under these conditions both the released and the cell surface-associated activities are estimated. The enhancement due to contact is illustrated in Figure 3. This increase in proteolysis due to contact is referred to as cell surface-associated enzyme activity, and it is obtained by taking the difference between the values obtained for "contact" and "no contact" incubation configurations. This activity may not be solely an expression of a membrane-bound enzyme since there might be a slightly higher concentration of released enzyme at the vicinity of the cell surface. In all experiments, the released enzymes were active for 3 to 6 h of incubation regardless of the cell passage studied. However, the rate of surface-associated activity always decreased after 60 to 90 min of incubation and leveled off beyond the second hour. This finding is not consistent with the interpretation that this activity is due to increased enzyme concentration in the proximity of the cell surface. Therefore we postulate the presence of an enzyme associ-

ated with the cell exterior, which has a finite probability of becoming detached under the assay condition. Preliminary experiments indicate that there is a decrease of surface-associated proteolytic activity with increasing serial cell passages. This gradual decrease resembles the loss of a critical neutral protease in aging WI-38 cells (12). Immmunological experiments are in progress to test whether the endothelial protease detected in this assay is identical to the plasminogen activator, urokinase, since previous studies have demonstrated fibrinolytic activity from these cells (13).

Increased proteolytic activity has been reported for invasive tumors of the human breast and colon (6). Recently we used this method to estimate cell surface-associated proteolytic activity on normal, carcinogen-treated and spontaneously-transformed rat liver epithelial cells (11) and demonstrated a significant increase in surface proteases with transformation. Endothelial cells, responsible for the controlled invasion of healing normal or neoplastic tissues to form a neovascular network, resemble the transformed cells in their increased capacity to hydrolize proteins covalently linked to a solid phase.

ACKNOWLEDGEMENTS

We express appreciation to Carole Kurahara, Csaba Csipke and Channing Der for their participation in these experiments. We are grateful to Mary Duda for the preparation of this manuscript.

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