Regulation of Plasminogen Activator Activity in Human Fibroblastic Cells by Fibrosarcoma Cell-derived Factors

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ABSTRACT

Low-molecular-weight protein factors (M, 8,000 to 18,000) from serum-free conditioned medium of human fibrosarcoma (8387) cells reversibly enhanced the secretion of proteinase-inhibitory activity by cultured normal human skin fibroblasts. This inhibitory activity could be absorbed by immobilized plasminogen activator (PA) of urokinase type but not by heparin, and it was sensitive to treatment with sodium dodecyl sulfate. The secretion of a heparin-binding M, 60,000 proteinase inhibitor, resembling protease nexin, was also detected. Early passages of adult skin fibroblasts do not contain or secrete PA. When cell types secreting this enzyme were tested, the fibrosarcoma-derived factors decreased the PA secretion detectable after sodium dodecyl sulfate treatment in all conditioned media of normal and malignant fibroblastic cells examined, including the 8387 cell line itself. However, no effects on the secretion of PA by normal or malignant cells of epithelioid origin or by melanoma cells were seen. A similar preparation from human epidermoid carcinoma (A431)-conditioned medium did not affect the PA activity or secretion of proteinase inhibitors from fibroblastic cells. The ability of sarcoma cells to modulate the production of PA inhibitors is a novel characteristic in the regulation of cellular proteolysis.

INTRODUCTION

Plasminogen is a proteinase zymogen widely distributed in plasma and other body fluids (5). When converted into active form, it is capable of hydrolyzing a large variety of proteins. The ability of plasmin to convert latent collagenases into active form creates lytic activity, degrading the components of pericellular matrix and basal laminas (31). Two functionally and structurally distinct plasminogen activators exist in the body, u-PA and t-PA, found mainly in urine and in circulation, respectively (5). These enzymes are, in turn, activated from a precursor protein by yet unknown extracellular mechanisms (34, 45). Several types of cells secrete PAs under culture conditions. In vivo, t-PA participates at least in the reactions of fibrinolysis, whereas the production of u-PA or uncharacterized PA activity has been clearly associated with various degradative phenomena, such as tissue remodelling during embryogenesis (46), cell migration (38), malignant transformation and tumor invasion (33, 37, 38), and processing of polypeptide hormones (13, 49).

The regulatory mechanisms of the PA-mediated proteolysis have recently attracted considerable interest. Close interactions between different types of cells seem to affect the synthesis rate of PAs (23, 27), and cells secrete factors modulating the release of PA by other cells (7, 11, 14, 25, 30). Inhibitors secreted by several cell types are capable of inhibiting directly the PA reaction. So far, the best characterized are protease nexins, heparin-binding components released by several types of human and other mammalian cells (20). PA inhibitors, obviously unrelated to protease nexin, have been described in human macrophages (41, 47), in rat hepatoma cells (12), and in human endothelial cells (26). The last one is obviously present also in circulation (22, 48).

The regulation of these PA inhibitors in poorly known. The secretion rate of the hepatoma cell inhibitor is modulated by glucocorticoids (12), and the secretion of protease nexin can be increased by treatment of the cultures with phorbol esters, epidermal growth factor, and thrombin (9). In the present study, we report that cultured human 8387 fibrosarcoma cells produce factors that are able to modulate the secretion of PA activity in cultures of fibroblastic cells, by both decreasing the secretion of PA activator and increasing the production of an inhibitory substance obviously unrelated to protease nexin. This inhibitor affects the PA activity in both the sarcoma cells themselves and normal human fibroblasts derived from embryonic tissues.

MATERIALS AND METHODS

Partial Purification of Sarcoma Cell-derived Proteins. Human 8387 fibrosarcoma cells (1) were grown to confluence in Medium 199 containing 10% heat-inactivated fetal bovine serum and washed 3 times with serum-free Medium 199, followed by a 12-h incubation in Medium 199 to remove the serum proteins of culture medium. Subsequently, the cells were incubated in Medium 199 for two 48-h periods, and the media were collected. The medium was clarified by centrifugation and concentrated 100-fold using an Amicon hollow fiber apparatus. The concentrate was dialyzed against 1 M acetic acid and lyophilized. The proteins were then extracted with a small volume of 1 M acetic acid followed by removal of insoluble material by centrifugation. Chromatography was carried out on a Bio-Gel P-30 column which was eluted with 1 M acetic acid (Chart 1). Fractions were lyophilized and dissolved in Medium 199 prior to addition on cells. Conditioned medium of cultured human epidermoid A431 carcinoma cells was prepared and fractionated in a similar way.

Test Cells. The cells used were either of local origin (adult skin fibroblasts, HES, HEL, HuA; see Table 1) prepared by conventional methods, or they were obtained from American Type Culture Collection (Rockville, MD) (WI-38, CCL 75; HT-1080, CCL 121; G-361, CRL 1424). In addition, human cell lines 8387 (1) and A431 were used (obtained from Dr. J. E. De Larco, National Cancer Institute, NIH, Bethesda, MD).

Cells were grown in plastic Linbro wells in Medium 199. When confluent, they were washed twice with Medium 199 and incubated for 6 h to remove serum proteins. The medium was substituted by fresh Medium 199, and an aliquot of each lyophilized column fraction, dissolved in Medium 199, was added. The Linbro wells were incubated at 37°C for 48 h, the medium was collected, and the cells were dissolved in 200 µl of 0.5% Triton X-100 in phosphate-buffered saline.

1 This work was supported by the Finnish Cancer Foundation, Nordisk Insulinfond, and the Academy of Finland.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; SDS, sodium dodecyl sulfate; PA, plasminogen activator.

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used in the PA assay described above. The inhibition of plasminogen activation (or of plasmin) was detected as an unlysed zone of casein.

RESULTS

Fibrosarcoma-derived Activity. Concentrated conditioned serum-free medium from human fibrosarcoma (8387) cells was prepared and fractionated in 1 M acetic acid (Chart 1). Fractions corresponding to M, 8,000 to 18,000 were observed to stimulate the PA-inhibiting capacity of normal human adult skin fibroblast-conditioned medium (Chart 2). The increase in PA inhibition was detected by adding a constant amount of u-PA to the fibroblast-

Chart 1. Gel permeation chromatography of 8387-conditioned medium. The conditioned medium was concentrated (see "Materials and Methods") and chromatographed in a Bio-Gel P-30 column eluted with 1 M acetic acid. The column fractions were then lyophilized, dissolved in serum-free medium, and analyzed. The protein profile (——) and PA activity (-----) are plotted. The molecular weight markers used were bovine serum albumin (M, 67,000), chymotrypsinogen (M, 25,000), RNase A (M, 13,700), and epidermal growth factor (M, 6,000). PU, Ploug units.

Caseinolytic PA Assay. PA was measured using a radial caseinolysis assay in plasminogen-containing agarose gel as described earlier in detail (40). Sample wells were punched in the gel and filled with 10 μl of the culture medium. During diffusion of the samples, a clear caseinolysis disc appeared as a result of the activation of plasminogen in the gel. When needed, a final concentration of 0.3% SDS was added to the samples. While this treatment irreversibly denatured the cell-produced inhibitors, it had no effect on the PA activity, which was recovered after diffusion of SDS in the gel. A human urokinase preparation (Calbiochem, La Jolla, CA) was used as a standard, and PA activity was plotted in Ploug units per ml (36). Plates without added plasminogen were included to control for non-PA proteolysis.

125I-Plasminogen-Plasmin Conversion by u-PA. Purified human plasminogen was iodinated according to the method of Krohn et al. (21). The conversion was carried out in a mixture containing 50,000 cpm of the 125I-plasminogen, proteinase inhibitor aprotinin (200 units/ml) (Trasylot; Bayer, Leverkusen, Federal Republic of Germany) to inhibit autocatalytic activity of the plasmin generated, and the test sample. Urokinase was added to a final concentration of 1 Ploug unit/ml. After a 6-h incubation, the samples were mixed 1:1 with sample buffer containing 4% SDS and 10% 2-mercaptoethanol and analyzed by electrophoresis on a discontinuous 3.5 to 8% polyacrylamide slab gel (24).

Assay Systems for the PA Inhibitor. The PA inhibitor-containing samples were analyzed using the agarose:casein plate assay as follows. The sample was mixed with an equal volume of the urokinase standard (1.0 Ploug unit/ml) and subsequently applied to the well. The inhibitory activity was detected by comparing the caseinolysis to that around control wells after application of the urokinase standard diluted similarly with phosphate-buffered saline, pH 7.4.

In the zymography inhibitor assay, the samples were electrophoresed in a 3.5 to 8% discontinuous SDS:polyacrylamide slab gel under nonreducing conditions. Small amounts of u-PA (0.1 Ploug unit/ml) were included in the washing fluid of the polyacrylamide gel, and the PA activity was visualized with an agarose:casein gel overlay similar to that

Chart 2. Effect of fractionated 8387-conditioned medium on the PA-inhibiting activity secreted by adult skin (AS) fibroblasts. Cells grown in plastic Linbro wells were incubated in serum-free medium for 24 h and washed, and subsequently, fresh serum-free medium with the sarcoma-conditioned medium fractions was added (final protein concentration, 1 μg/ml). The cultures were incubated for 48 h, and the medium was collected, clarified by centrifugation, and analyzed. The inhibitory activity in the culture medium was demonstrated by adding a constant amount of urokinase [1 Ploug unit (PU)/ml] to the samples. The uninhibited activity was then measured by the caseinolysis assay in the absence of SDS. The sharp peak of PA activity in Fractions 20 to 23 results from the added, 8387 cell-derived PA, as shown in Chart 1 (-----), and the position of this sarcoma:PA (8387-PA). The level of urokinase activity is indicated on the left (Arrow B).

Chart 3. Effect of fractionated 8387-conditioned medium on the PA activity secreted by embryonic skin fibroblasts (HES). The experiment was carried out as described in the legend for Chart 2. The samples were analyzed in the caseinolysis assay in the presence of SDS. The sharp peak of PA activity in Fractions 20 to 23 results from the added, 8387 cell-derived PA, as shown in Chart 1 (-----), and the position of this sarcoma:PA (8387-PA). The background secreton of PA by HES cells is shown on the left (Arrow B). PU, Ploug units.
conditioned medium. PA activity, not neutralized by the inhibitor, was quantitated using a caseinolysis assay and indicated in Ploug units. Conditioned medium of early passage adult skin fibroblasts does not contain PA activity measurable by the caseinolysis or zymography assays (42). In cultures of embryonic fibroblasts, the 8387 cell-derived factors significantly decreased the accumulation of cell-secreted PA activity in the culture medium (Chart 3).

Fractions of the fibrosarcoma-conditioned medium were active on adult skin fibroblasts at protein concentrations as low as 100 ng/ml (Chart 4). No inhibitory activity of the column fractions themselves could be demonstrated in the caseinolysis assay, even at concentrations of 20 µg/ml. When the active 8387 fractions and the conditioned medium from adult skin fibroblasts were mixed and incubated at 37°C for 2 h, no inhibition of added u-PA was seen. This indicated that the observed effect was not due to a direct interaction between proteins but was mediated through the fibroblasts.

The effect of fibroblasts was fully reversible, and a situation comparable to that in the control cultures was attained after washing away the effective 8387 cell-conditioned medium fractions. The effect of the 8387 cell-derived factors was time and dose dependent (Charts 4 and 5).

Properties of the Sarcoma-derived Factors. The 8387 cell-derived activity was destroyed by boiling for 10 min or by incubation with trypsin (1 µg/ml) for 30 min at 37°C, the latter suggesting a proteinaceous nature to the factor(s). It was partly destroyed by incubation at 56°C for 60 min but was resistant to pH 2.0 for 60 min. The inhibitor-inducing effect was lost when the fractions were preincubated with 1 mM benzamidine (final concentration in the cell culture, 0.02 mM). Reduction with 5% 2-mercaptoethanol (without heating) followed by removal of the reactant with lyophilization also abolished the activity, suggesting that disulfide bonds are essential for the activity. Polyacrylamide gel electrophoresis of the active fractions followed by protein staining showed that the fractions contained several low-molecular-weight polypeptides.

Characterization of the Induced PA Inhibitors. The zymography inhibitor assay revealed a M, 60,000 polypeptide band (Fig 1) in skin and lung fibroblast cells and media, which was completely removed by absorption with heparin:Sepharose in the presence of 0.01% Tween-80. However, although the M, 60,000 polypeptide was slightly increased after treatment of the cells with the 8387 cell-derived fractions, its removal with heparin:Sepharose did not simultaneously remove the u-PA-inhibiting activity from the adult skin fibroblast-conditioned medium (Fig. 2). This was accomplished with absorption by immobilized u-PA, which in turn did not totally remove the M, 60,000 band. The PA-inhibiting property of the adult skin fibroblast-conditioned medium factor was also demonstrated in the 125I-plasminogen conversion assay (Fig. 3). These findings suggested that another inhibitory function apart from the heparin-binding protease:nexin-like activity is present in the medium, although the sensitivity of
PA AND SARCOMA-DERIVED FACTORS

Fig. 1. Zymographic inhibitor assay of the adult skin fibroblast culture. Lane 1, cell lysate (cells taken in phosphate-buffered saline containing 0.5% Triton X-100) with the M, 60,000 inhibitory activity; Lane 2, conditioned medium of the adult skin fibroblasts; Lane 3, absorption with immobilized heparin removed the activity, while immobilized u-PA (Lane 4), gelatin (Lane 5), or IgG (Lane 6) did not affect the M, 60,000 band. Elution of the respective absorbants with sample buffer revealed the M, 60,000 polypeptide attached to heparin (Lane 7), but not to u-PA (Lane 8), gelatin (Lane 9), or IgG (Lane 10). The samples were analyzed in SDS:polyacrylamide gels followed by casein:agar gel overlay (see "Materials and Methods"). The agar gel shown was photographed under indirect illumination.

Fig. 2. Demonstration of the sarcoma cell-induced inhibitor after heparin absorption in adult skin fibroblast-conditioned medium. Lane 1, conditioned medium collected after treatment of the cells with the sarcoma cell factor (Fraction 43 from Chart 1, 1 μg/ml). Lane 2, corresponding medium from untreated cultures. Lane 3, conditioned medium from sarcoma cell factor-treated cultures absorbed with heparin-Sepharose. Lane 4, corresponding untreated medium absorbed with heparin-Sepharose. Line a, the above-described samples. Line b, urokinase added to the samples (1 Ploug unit/ml). Line c, 0.3% SDS added to the samples. Line d, SDS and urokinase added to the samples. UK, urokinase control (amount added to samples of Lines b and d). Note that absorption with heparin-Sepharose removes only a small part of the sarcoma cell factor-induced inhibitory activity. This inhibitory activity is abolished by SDS.

Fig. 3. Effect of adult skin fibroblast-conditioned medium on the activation of 125I-plasminogen. The fibroblast cultures were treated with 8387 Fraction 43 (Chart 1) for 48 h, and the collected fibroblast-conditioned medium was absorbed with immobilized heparin to remove the M, 60,000 polypeptide. For inhibition of the u-PA activity, the characteristic cleavage of the M, 60,000 plasminogen molecule to the M, 56,000 and 25,000 polypeptides by u-PA (Lanes 3 and 4) did not take place in the presence of the conditioned medium (Lanes 1 and 2). Similar inhibition of u-PA was caused by 3 mw benzamidine (Lanes 5 and 6). All the reaction mixtures contained the proteinase inhibitor aprotonin (200 IU/ml) to prevent autodigestion of plasmin and 1 Ploug unit of u-PA per ml. The samples were analyzed in duplicate in a discontinuous 3.5 to 8% SDS:polyacrylamide gel slab under reducing conditions and visualized by autoradiography.

The inhibitory activity was sensitive to treatment with SDS but not with TX-100 or Tween-80. This was verified by the addition of u-PA to adult skin fibroblast-conditioned medium. All added PA activity was recovered after addition of 0.3% SDS. It thus appears that low concentrations of SDS (Fig. 2) are sufficient to prevent the inhibitory function in the caseinolysis disc assay. The M, 60,000 inhibitory activity was readily visualized in the SDS gel zymography assay after removal of the detergent, which suggests a reversible effect of SDS on at least this inhibitor. Acid treatment (pH 2.0, 30 min) destroyed the inhibitors, as well as heating at 56°C for 60 min.

Effects on Different Types of Cells. In order to study the extent of the phenomenon in other cell systems, we tested the ability of the active fractions to decrease PA activity on a series of normal and malignant cell types under serum-free conditions (Table 1). A common feature was the positive effect on all fibroblastic cell lines, both normal and malignant. HT-1080, originally considered to be a fibrosarcoma cell line, was an exception. It has, however, recently been demonstrated that HT-1080 cells secrete basement membrane collagen and laminin, indicating an epithelial or endothelial rather than mesenchymal phenotype (2). Cell types other than fibroblasts (epithelial and melanoma cells) did not respond to the 8387 cell-derived factors.

Most of the cell types examined secrete plasminogen activators, which makes it difficult to distinguish between a decrease in the PA synthesis and an increase in the inhibitor synthesis.
The human fibrosarcoma cell line (8387) has been shown to produce a family of growth-promoting polypeptides (8). These factors have been purified, and they resemble the insulin-like growth factors (29). The 8387 cells do also produce into their media proteolytic activity which is able to affect cell-associated fibronectin (16). This latter activity was associated with a M, 10,000 column fraction, and its activity was inhibited by proteinase inhibitors. A major target of the M, 10,000 peptides was a cellular M, 66,000 protein, which was cleaved to a M, 62,000 form (17). A similar cleavage was brought about by thrombin (18) and by affinity-purified urokinase (19), indicating that the M, 66,000 protein is a target for a number of proteinases in cultured cells. The preparation of the 8387-conditioned medium used in the present study obviously contains the above-described peptides.

There is increasing evidence that the metabolism of u-PA involves secretion of the enzyme in inactive proactivator form (34, 45), activation by so far uncharacterized extracellular mechanisms, inactivation by specific proteinase inhibitors, and uptake into cells by a receptor-mediated endocytosis (3, 15, 44). Several PA inhibitors secreted by cultured cells have been identified recently (4, 10, 12, 26, 28, 41, 42, 47). Such inhibitors have also been found in vivo (22, 32, 39, 48). So far, the best characterized one is the protease nexin, a M, 51,000 polypeptide, functionally related to circulating Antithrombin III (43). The presence of protease nexin in fibroblasts, its ability to bind to heparin, and molecular weight are properties resembling the M, 60,000 protein inhibitor seen in our zymography inhibitor assay. Recovery of activity after treatment with SDS and denaturation in acidic conditions are also shared by the inhibitors. Whether the M, 60,000 inhibitor interacts directly with u-PA is not known. It appears that there are at least 2 different PA inhibitors present in the medium of cultured fibroblasts. The heparin-binding inhibitor seen in the zymography assay does not bind to immobilized u-PA, and its removal by heparin has only a weak effect on the total inhibitory activity of the conditioned medium from treated cells towards u-PA-mediated plasminogen activation. The more precise character of this PA-inhibiting capacity not bound by heparin is not known.

The secretion of PA by different cells appears to be very sensitive to various stimuli. It is, however, often difficult to distinguish the inhibitory effect from decreased production of PA. Stimulation of PA inhibitor production has been described after treatment with glucocorticoids, at least in cultured fibroblasts and hepatoma cells (6, 12). In the present study, the inhibiting activity of the adult skin fibroblast-conditioned medium against exogenous u-PA was abolished in the presence of SDS. We thus presume that the decrease in PA activity in the other fibroblastic cells, detected in the zymography and caseinolytic disc assay, was actual and not due to induction of a detergent-resistant proteinase inhibitor. The possible existence of such an unusually stable PA inhibitor complex (28) as an explanation for the decreased PA activity cannot be ruled out with the methods used.

Interactions between different types of cells resulting in alterations of the synthesis rate of PA have been reported in a few cases. Macrophages increase the production of PA by synovial fibroblasts (14), and malignant cells stimulate the PA activity of normal skin fibroblasts (7). It has been reported that t-PA secreted by melanoma cells is removed from culture medium at a constant rate by normal fibroblasts (15), possibly through interaction of t-PA with matrix components known to be taken up by the cells. The protease nexin mediates the uptake of u-PA(s) and other serine proteinases in fibroblasts. We found a decrease in the
extracellular PA activity, while the intracellular level of PA remained almost constant during the experiments. This could result from a direct effect of the fibrosarcoma factor on the secretion of the activator by fibroblasts.

The ability of sarcoma cells to affect the secretion of PA and to induce PA-inhibitory responses in fibroblastic cells is a novel feature in the regulation of PA activity. This kind of control of proteolysis may be involved in the progression of tumor growth in vivo, e.g., the selective resistance of tissues to metastatic growth of certain tumors could result from the local inhibition of PA activity which in turn may be a prerequisite to the development of metastases (35). These inhibitors may also be produced at certain stages of normal growth and differentiation.

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