Estradiol Preferentially Enhances Extracellular Tissue Plasminogen Activators of MCF-7 Breast Cancer Cells*

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Thomas J. Ryan, James I. Seeger, S. Anand Kumar, and Herbert W. Dickerman

From the Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201

MCF-7 human breast cancer cells secrete two immunologic types of plasminogen activator, one related to urokinase, the other unrelated. We have now examined whether estrogen stimulation of secreted plasminogen activator activity reflects an increase in one or both types. Examined semiquantitatively by sodium dodecyl sulfate-polyacrylamide gel electrophoretic zymography, the conditioned media of control cells were seen to contain a major activator band (M, 54,000) immunologically related to urokinase and a barely discernible doublet (M, 64,000 and M, 68,000). Addition of estradiol or, at much higher concentrations, testosterone led to marked enhancement of doublet activity, while the 54-kDa band was invariant. The 64-68-kDa doublet was immunoreactive with rabbit antiserum directed against Bowes melanoma tissue activator and monoclonal antibodies. Enhancement of doublet activity was correlated with hormone-induced increases in total secreted plasminogen activator activity. Neither progesterone nor dexamethasone increased total activity or the 64-68-kDa zones of lysis. Estradiol and testosterone alterations were blocked by appropriate concentrations of an estrogen antagonist (LY156758), actinomycin D, or cycloheximide. Regulation of MCF-7 cell-secreted tissue plasminogen activators thus appears to be mediated by an estrogen receptor process and to require sustained RNA and protein synthesis.

MCF-7, a stable cell line of human metastatic breast cancer origin, is responsive to estrogen. It contains cytosol estrogen receptors which are capable of translocation to the nucleus (1) and, in the presence of hormone, of selectively changing the cell’s protein synthesis (2, 3). A possible example of such changes is an estrogen-dependent rise in extracellular plasminogen activator activity; the increase is sensitive to RNA-and protein-synthesis inhibitors (4).

Multiple forms of secreted plasminogen activators of fetal and tumor cell origin are immunologically distinct (5, 6). The plasminogen activators secreted by MCF-7 cells are of two immunologic types, one related to urokinase, the other unrelated (7). This distinction led us to postulate that these activators are regulated by separate mechanisms. Under the experimental conditions of the present study, addition of physiologic concentrations of E2 to MCF-7 cells preferentially enhanced the activity of tissue plasminogen activator-like proteases with apparent molecular weights of approximately 64,000 and 68,000, while the urokinase-like activator of M, 54,000 showed no change.

MATERIALS AND METHODS

An established MCF-7 cell line was obtained from the E. G. and G. Mason Institute, Rockville, MD, and cultured in plastic tissue-culture flasks at 37°C in 5% CO2. For maintenance Dulbecco’s minimal essential medium was supplemented with 10% heat-inactivated horse serum, streptomycin (100 µg/ml), penicillin (100 unit/ml), insulin (1 µg/ml), L-glutamine (2 mM), and nonessential amino acids.

The experimental growth schedule was as follows: After plating at 104 cells/100 X 20-mm flask, the cells were cultured on days 0-2 in the maintenance medium containing 10% horse serum, on days 3-5 in serum-free medium, and on days 6 and 7 in serum-free medium supplemented with individual hormones, the antiestrogen LY156758, or controls with the ethanol vehicle alone. Finally, the cells were sustained in medium alone for an additional 20 h unless otherwise indicated. The cells were counted manually and nonviable cells estimated by trypan blue staining.

The final culture medium (designated the conditioned medium) was 10% culture flasks. After centrifugation at 1000 X g x 10 min, each portion was dialyzed against 0.01 M ammonium acetate (pH 8.5). Total plasminogen activator activity was assayed by the human plasminogen-dependent hydrolysis of Cbz-Lys-BzL using the spectrophotometric method of Coleman and Green (6).

Rabbit antiserum (to urokinase) (9), prepared as described previously (7), was purified by chromatography on protein A-Sepharose. Goat antiserum to Bowes melanoma cell tissue plasminogen activator was purchased from Bio Pool, Hornefors, Sweden. Plasminogen was isolated from human Cohn Fraction III paste by lysine-Sepharose chromatography (10).

The molecular heterogeneity of the plasminogen activators was determined by using a modification of the SDS-PAGE zymography technique of Huessen and Dowdle (11). For immunologic characterization of the plasminogen activator, rabbit antiurokinase IgG or goat antiserum prepared against Bowes melanoma tissue activator was copolymerized with sodium dodecyl sulfate, casein, and plasminogen according to the method of Camilo et al. (12). Protein was measured by the method of Bradford (13). Total plasminogen activator activity is given as CTA milliunits/106 MCF-7 cells.

RESULTS AND DISCUSSION

Marked increases in MCF-7 cell-secreted plasminogen activator activity were observed by Butler et al. (4) when E2 was added to the cultures over a concentration range of 10-8 to 10-6 M. Our study, using a different growth schedule and a spectrophotometric assay, based on the plasminogen-dependent hydrolysis of Cbz-Lys-BzL, confirmed the E2 stimulation of this activity over the same range with a peak at 10-7 M (Fig. 1). Testosterone also increased plasminogen activator activity, but it required concentrations 100-fold greater than E2.

To determine which molecular species of secreted activators were associated with the E2-induced rise in activity, aliquots

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of the dialyzed conditioned media were analyzed by SDS-PAGE zymography (Fig. 2). Conditioned medium from control cells had a major activator band of 54 kDa, coincident with the major band of urokinase, as well as traces of lytic trol cells had a major activator band of 54 kDa, coincident bands at 81, 68, 64, and 33-34 kDa (Fig. 4 lane 2). The zymograms of media from E2-treated cells were markedly different. Distinct plasminogen activator bands at 64 and 68 kDa increased in intensity at hormone concentrations of $10^{-10}$ M and higher, while the 54-kDa band was constant at all E2 concentrations. In gels prepared without plasminogen there were no areas of caseinolysis.

The immunologic reactivity of the activators was assessed by zymography with antiurokinase IgG in the gels (Fig. 2B). Under these conditions, the bands in the urokinase standard (Fig. 2B, lane 1) were absent, as were all bands found in the conditioned media except the 64-68-kDa doublets and traces of the 81-kDa bands. These results indicate that the 64-68-kDa bands are immunologically unrelated to urokinase.

Zymography is semiquantitative, so we shall use the term enhancement to designate the increased intensities of the doublet lytic bands. The hormone specificity of enhancement was examined by exposing cells for 48 h to $10^{-8}$ M concentrations of E2, dexamethasone, or progesterone. When analyzed for plasminogen activator heterogeneity, only conditioned media from E2-treated cells had enhanced 64-68-kDa doublet activity (Fig. 3A). The 54-kDa band was invariant regardless of culture conditions. Furthermore, only conditioned media obtained from E2-treated cells demonstrated significantly elevated total plasminogen activator activity as measured by plasminogen-dependent Cbz-Lys-Bzl hydrolysis (see the legend to Fig. 3).

The immunologic relationships of the secreted plasminogen activators were visualized by zymography with gels that contained antiurokinase IgG (Fig. 3B) or goat antiserum against Bowes melanoma tissue plasminogen activator (Fig. 3C). In gels containing antiurokinase IgG, all bands except the 64-68-kDa doublet and traces of 81-kDa lysis were eliminated. The doublet bands, although faint, were present in conditioned media from the control, dexamethasone-, and progesterone-treated cells. In gels containing antiserum to tissue plasminogen activator the 64-68-kDa doublet was removed and the 54-kDa band was present in all media. These data indicate that the 64-68-kDa doublet, enhanced or not, is immunologically related to tissue plasminogen activator and not to urokinase. Testosterone also induced greater plasminogen activator activity but only at much higher concentrations (Fig. 1). At the effective concentrations, testosterone led to a
similar enhancement of 64-68-kDa doublet activity (data not shown).

This dual effect of estrogen—inducing total plasminogen activator activity and enhancing the intensity of the tissue plasminogen activator-like doublet—was further examined by culturing cells with E₂ in the presence or absence of the benzothiophene-derived estrogen antagonist LY156758 (14). The inhibitory effect of LY156758 on induction (Table I) was consistent with an antagonist effect on an estrogen-mediated process at concentrations of LY156758 10-fold greater than E₂. When the conditioned media from this experiment were examined by SDS-PAGE zymography, the enhanced doublet associated with E₂ treatment was suppressed at antagonist levels 10-fold greater and eliminated at levels 100-fold greater than E₂ (Fig. 4A). The media from cells exposed to LY156758 alone did not show the 64-68-kDa doublet. In no case, was the 54-kDa band altered by the presence of E₂, LY156758, or combinations of both. Zymography in gels prepared with antiurokinase IgG also demonstrated the enhanced nonurokinase bands in cells exposed to E₂ alone or combined with equimolar LY156758, and, at higher antagonist concentrations, there was again a reduction or loss of detectable activity (Fig. 4B). Similar effects were observed with testosterone as an inducer of activity and enhancer of the nonurokinase doublet, except that doublet enhancement was eliminated at an antagonist concentration only ¼ that of testosterone (10⁻⁷ and 10⁻⁸ M, respectively). These data suggest that E₂ alters the distribution of MCF-7 cell-secreted plasminogen activators by a receptor-mediated process and that testosterone mimics this effect by binding to estrogen receptors (15).

To determine the effect of sustained RNA or protein synthesis on the secretion of multiple forms of plasminogen activator, control and E₂-treated cells were exposed to either actinomycin D (0.8 μg/ml) or cycloheximide (15 μg/ml) during the second day of hormone treatment. Under these conditions, actinomycin D led to >90% inhibition of uridine incorporation into acid-insoluble material, and cycloheximide inhibited amino acid incorporation by >75% while cell death did not exceed 6% of total cells.

The effects of these inhibitors on plasminogen activator activity are shown in Table II. Actinomycin D markedly reduced the production of secreted activators by both control and E₂-treated cells. When cycloheximide was added for day 7 of cultivation and then removed for preparation of the conditioned media, the total activity in medium from the control cells increased significantly, while activity in medium from E₂-treated cells was moderately reduced. In contrast, when cycloheximide was retained through the final culture period, the activity secreted by E₂-treated cells fell to that of the control cells. Zymography of media from actinomycin D-inhibited control and E₂-treated cells indicated a general decrease in lytic activity but no change in the distribution of secreted activators. Cycloheximide inhibition, however, resulted in a complete and selective loss of the 64-68-kDa doublet activity.

The present study thus confirms the previously noted estrogen-induced rise in MCF-7 cell-secreted plasminogen activator activity (4, 16-18) and provides evidence that the increase is associated with enhancement of molecular species of activators which are immunologically related to tissue plasminogen activators rather than urokinase. Although SDS-PAGE zymography is a semiquantitative technique, it is apparent that the 64-68-kDa doublet zones of caseinolysis are greater at concentrations of E₂ and testosterone which increase total plasminogen activator activity and that the zones are reduced selectively at the effective concentrations of LY156758 and cycloheximide. These findings indicate that enhancement is an estrogen receptor-mediated process which requires sustained RNA and protein synthesis.

Identification of two immunologic types of MCF-7 cell-secreted plasminogen activators (7) suggested the possibility that these activator types were modulated in separate ways. The present study demonstrates that, at least as far as estrogen effects on these cells are concerned, separate regulation of activator activity does occur.

**TABLE I**

| Concentration | Activity |
|---------------|----------|
| LY156758      | E₂       | CTA units (× 10⁶)/10⁶ cells |
| M             |          |
| 10⁻⁸          | 12.8     |
| 10⁻⁷          | 22.9     |
| 10⁻⁶          | 20.7     |
| 10⁻⁵          | 10.7     |
| 10⁻⁴          | 9.1      |
| 10⁻³          | 9.5      |
| 10⁻²          | 8.3      |

*Fig. 3. Effect of E₂, progesterone, and dexamethasone on molecular heterogeneity of MCF-7 cell-secreted plasminogen activators (A) with no added antibodies, (B) in the presence of rabbit antiiurokinase IgG (11.4 μg/ml), and (C) in the presence of goat antiserum to Bowes melanoma tissue plasminogen activator (264 μg/ml). The procedures for growth and gel preparation are described under "Materials and Methods." The steroids were present at 10⁻⁸ M during days 6 and 7 of cultivation. The samples (and their activities in CTA milliunits/10⁶ cells) are: lane 1, urokinase; lane 2, control (8.7); lane 3, progesterone-treated (9.9); lane 4, E₂-treated (18.9); lane 5, dexamethasone-treated (9.1); lane 6, E₂-treated (18), and lane 7, plasmin.*
The cells were exposed to E2 (10^{-8} M) or vehicle alone for 48 h (days 6 and 7 of cultivation). Actinomycin D (0.8 μg/ml) or cycloheximide (15 μg/ml) was added for day 7 of cultivation. In some experiments, the inhibitor was removed for the final 20 h of cultivation; in other experiments cycloheximide was retained. Nonviable cells were determined by trypan blue staining.

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