Communication

Regulation of Urokinase-type Plasminogen Activator Production by Cultured Human Cytotrophoblasts*

(Received for publication, April 30, 1987)
John T. Queenan, Jr., Lee-Chuan Kao, Carlos E. Araujo, Alfredo Olsos-Aguirre, Thaddeus G. Golos, Douglas B. Cines, and Jerome F. Strauss III

From the Departments of Obstetrics and Gynecology, Medicine and Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Cultured human cytотrophoblasts synthesize and secrete urokinase-type plasminogen activator (uPA) during the first 24 h of culture, but secretion declines during the subsequent day. In contrast, synthesis and secretion of fibronectin increases during the 2 days of culture. The levels of uPA mRNA parallel the changes in synthesis and secretion of uPA. Treatment of cytотrophoblasts with 8-bromo-cAMP (1.5 mM) transiently raises uPA mRNA levels and uPA secretion. This treatment reduces fibronectin mRNA levels and causes a sustained increase in β chorionic gonadotropin mRNA content and chorionic gonadotropin secretion. We conclude that a cAMP-mediated process up-regulates uPA expression in cytотrophoblasts. However, the stimulatory effect of the cyclic nucleotide analog on uPA is transient.

Urokinase-type plasminogen activator (uPA)* is believed to play an important role in the degradation of extracellular matrix in diverse processes including implantation of the trophoblast in the uterine wall (1, 2). uPA is synthesized and secreted as a ~50,000 single chain protein which is converted by limited proteolysis to a fully active enzyme consisting of two amino acid chains joined by a disulfide bond (1). A M, ~30,000 form of uPA, which is a partial degradation product of the M, ~50,000 enzyme, also maintains catalytic activity. The production of uPA by various tissues is under hormonal control, and agents which elevate cellular cAMP levels can increase uPA synthesis by promoting transcription of the uPA gene (3). Studies which implicate PA in the process of trophoblast implantation have been carried out in the mouse (2), but little is known regarding PA production by human trophoblast. We have developed a method to isolate cytотrophoblasts, the cells which invade the uterus, from human placenta (4). These cytотrophoblasts respond to the analog of cAMP, 8-bromo-cAMP, by increasing production of chorionic gonadotropin (βCG) and progesterone (5, 6). Here we report that the cultured cytотrophoblasts synthesize and secrete uPA in an evanescent fashion, and that 8-bromo-cAMP acutely increases the synthesis and secretion of uPA by elevating uPA mRNA.

**MATERIALS AND METHODS**

Preparation and Culture of Cytotrophoblasts—Cytотrophoblasts were isolated from term placents obtained following normal spontaneous vaginal delivery or uncomplicated cesarean section, as previously described by Kilman et al. (4). This procedure yields a highly purified (≥98% pure) preparation of cytотrophoblasts with ≥90% viability. Briefly, villous tissue was subjected to three 30-min digestions with 0.125% trypsin and 0.2 mg/ml DNase I (Sigma). Collected cells were applied to a 5-70% Percoll gradient. After centrifugation at 1200 × g, the middle band (density 1.048-1.062 g/ml) containing the cytотrophoblasts was removed, washed, and diluted to a concentration of 1 × 10⁶ cells/ml with Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 25 mM HEPES, and 50 mg/ml gentamycin (DMEM-HG). In experiments in which uPA synthesis and mRNA were quantitated, the medium was supplemented with 20% heat-inactivated fetal calf serum, whereas in experiments in which uPA activity was determined, cells were cultured in serum-free medium on dishes coated with type I collagen (Sigma). Cell suspensions were plated into 35-mm Nuncells (Nunc, Roskilde, Denmark) culture dishes (1 × 10⁶ cells in 2 ml of medium) and incubated in humidified 5% CO₂, 95% air at 37 °C. 8-Bromo-cAMP (1.5 mM, Sigma) was added at the time of plating to some cultures. This concentration of 8-bromo-cAMP consistently stimulates hCG and progesterone secretion by the cytотrophoblasts (5, 6). Media were changed after 24 h and experiments were terminated after the second 24-h period. At termination, cells were harvested by scraping with a plastic spatula. Each experiment was repeated on at least three separate occasions using different cell preparations.

Detection of uPA Activity—Serum-free media from control and 8-bromo-cAMP-treated cell cultures were analyzed by zymography using the method of Gravelli-Piperno and Reich (7) as modified by Heussen and Dowdle (8). Media were mixed with an equal volume of 2 × Laemmli (9) sample buffer and electrophoresed in 10% polyacrylamide slab mini-gels (Idea Scientific, Corvallis, OR). Each experiment involved two gels, the first contained 0.1% gelatin to detect non-plasminogen-dependent proteases, and the second contained gelatin and purified human plasminogen (Sigma) to detect plasminogen-dependent proteases. Molecular weight standards (Bethesda Research Laboratories, Bethesda, MD), purified uPA (Sigma), and recombinant tissue plasminogen activator (tPA), generously provided by Genentech Corp. (San Francisco, CA), were applied to each gel. Electrophoresis was carried out at 4 °C for 2.5 h. Following SDS-PAGE, gels were treated for 1 h with 2.5% Triton X-100 in 50 mM Tris-HCl, pH 8.0 at room temperature to remove the SDS. The gels were washed three times for 1 h with 50 mM Tris-HCl, pH 8.0, and then incubated for 16 h at 37 °C, fixed, and stained with 0.1% Coomassie Brilliant Blue R-250.

Metabolic Labeling of Secreted uPA and Fibroeconitin with [35S]Methionine—Two hours before labeling with [35S]methionine, the serum-containing media were removed and replaced with an equal volume of serum- and methionine-free DMEM-HG. [35S]Methionine (100 μCi/ml, Du Pont-New England Nuclear) was then added, and the cells were incubated for 2 h. Media were collected and cells harvested. Equal volumes of culture medium (100 μl) from control and 8-bromo-cAMP-treated cultures were employed for immunoprecipitation of uPA using 250 μg of an IgG fraction of an antisemum raised in

---

* This work was supported by United States Public Health Service Grants HD-06274, HL-34044, and HL-0096 and grants from the Rockefeller and Mellon Foundations. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Obstetrics and Gynecology, Hospital of the University of Pennsylvania, Philadelphia, PA 19184.

‡ The abbreviations used are: uPA, urokinase-type plasminogen activator; hCG, human chorionic gonadotropin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; tPA, tissue plasminogen activator.
rabbits against human uPA (Green Cross, Osaka, Japan). As a control, immunoprecipitations with an IgG fraction of an antisera raised in rabbits against recombinant human tPA were performed. The immunoisolation procedure employed Pansorbin (Behring Diagnostics) to precipitate the immunoglobulin (10). The specificity of the uPA antibody was assessed by performing immunoisolations in the presence of 20 μg of purified uPA (Abbott). Fibronectin was immunoisolated from the medium using 20 μl of a rabbit anti-human fibronectin antibody (Behring Diagnostics) and protein A-Sepharose CL-4B to precipitate the IgG-bound fibronectin (11). Immunoprecipitation of labeled fibronectin could be prevented by the addition of 200 μg of plasma fibronectin (Biomedical Technologies, Inc., Cambridge, MA) to the immunoprecipitation mixture. The immunolabeled peptides were solubilized in Laemmli buffer (9), heated at 100 °C, and subjected to SDS-PAGE using 7.5% (uPA) or 5% (fibronectin) polyacrylamide gels in the presence of 2-mercaptoethanol (9). 35S-Labeled molecular weight markers were run on each gel. Following electrophoresis, gels were fixed for 1 h, impregnated with 20% trichloroacetic acid, and then placed in bags with 5-8 ml of a solution containing sodium salicylate, 1% glycerol, dried, and stored in contact with Kodak X-Omat film (Eastman Kodak, Rochester, NY) at −70 °C for autoradiography.

**RNA Isolation, Electrophoresis, and Blot Hybridization**—RNA was isolated from cells using the guanidine isothiocyanate-cesium chloride method (12). Equal amounts of RNA (10 μg), quantified by absorption at 260 nm, were denatured in MOPS/formaldehyde and electrophoresed in 0.8% agarose/formaldehyde gels and transferred to Nytran paper (Schleicher & Schuell) by standard procedures (12). A HindIII digest of bacteriophage λ DNA was also electrophoresed in these gels to estimate nucleic acid size.

 Autoradiograms with nick-translated cDNA probe were performed according to the method of Berent et al. (13). Dried filters were first moistened with 6× SSC (1× SSC = 0.15 M NaCl and 0.01 M sodium citrate), and then placed in bags with 5–8 ml of prehybridization solution (50% formamide, 5× SSC, 0.1% each of Ficoll, polyvinylpyrrolidone, bovine serum albumin, SDS, and 250 mg/ml denatured salmon sperm DNA) and incubated at 42 °C for 4 h. This solution was replaced with fresh solution to which was added nick-translated cDNA (5×106 cpm/ml) and nick-translated λ DNA. Hybridizations were continued for 18 h. Following hybridization, the filters were washed twice for 30 min at room temperature in 2× SSC and 0.1% SDS, followed by two 45-min washes at 65 °C in 1× SSC and 0.1% SDS. After the filters were blotted, wrapped in plastic, and placed with x-ray film (Kodak X-Omat) for autoradiography at −20 °C. Autoradiograms were scanned with a Kontes densitometer (Vineland, NJ) to determine relative changes in mRNA levels.

**Plasmid Preparation, Isolation, and Nick Translation**—A plasmid containing a 669-base pair cDNA for human uPA from pHUK-1 (14) was generously provided by Dr. Francesco Biasi (International Institute of Genetics and Biophysics, Naples, Italy). The cDNA was inserted into vector SP64 at the PstI/EcoRI site. A plasmid harboring a 579-base pair cDNA for hCG β-mRNA (15) was kindly provided by Dr. John Fiddes (California Biotechnology, Mountain View, CA). Another plasmid containing a 1300-base pair cDNA corresponding to a portion of the 3'-coding sequence and 3'-noncoding sequence of human fibronectin (16) was obtained from Dr. Mon-Li Chu, Thomas Jefferson School of Medicine, Philadelphia, PA. Probes were nick-translated with reagents obtained from Bethesda Research Laboratories (Gaithersburg, MD) with [35S]dCTP (Amersham Corp.) to specific activities of 1.5–3 X 1010 cpm/μl.

**hCG Assay**—hCG secreted into the incubation medium was quantitated with a commercially available radioimmunooassay (Seron Diagnostics, Braintree, MA). This assay is calibrated to the First International Reference Preparation.

**RESULTS**

**uPA Secretion and Synthesis by Culture Cytotrophoblasts**—Cultured cytotrophoblasts secreted a plasminogen-dependent protease during the initial 24 h of culture, but enzymic activity declined during the subsequent 24 h of incubation to approximately one-third of that released during the 1st day (Fig. 1). The major plasminogen-dependent activity migrated with a Mr value of ~50,000. No proteolytic activity with M r values corresponding to Mr, ~50,000 uPA or Mr, ~70,000 tPA was detected when plasminogen was omitted from the polyacryl-
FIG. 2. Metabolic labeling of secreted uPA and fibronectin.
A, synthesis and secretion of uPA. One-day-old cytotrophoblast cultures (1 x 10⁶ cells/well) were labeled for 2 h with [³⁵S]methionine as described in the text. Medium was subjected to immunoprecipitation with an IgG fraction of a rabbit anti-uPA antiserum in the absence (lane 1) or presence (lane 3) of uPA, or with an IgG fraction of a rabbit anti tPA antiserum (lane 2). Immunoprecipitates were subjected to SDS-PAGE under reducing conditions. A representative fluorograph is shown.

B, synthesis of uPA and fibronectin by cultures of cytotrophoblasts. Cytotrophoblast cultures (1 x 10⁶ cells/well) were labeled for 2 h with [³⁵S]methionine after 24 or 48 h of culture. Equal volumes of media were subjected to immunoisolation of uPA and fibronectin followed by SDS-PAGE (7.5% acrylamide for uPA, 5% acrylamide for fibronectin). The photographs are of the M, -50,000 region of the gels for uPA and the M, -220,000 region of the gels for fibronectin. The results are representative of four separate experiments.

hybridization analyses of total RNA extracted from cultured cytotrophoblasts with a specific uPA cDNA probe revealed the presence of the 2.5-kilobase mRNA at 24 h and a 70 ± 0.07% (X ± S.E., n = 3) reduction in this mRNA after 48 h of culture (Fig. 3). In contrast, the ~7.8-kilobase fibronectin mRNA increased 3-fold between 24 and 48 h of culture. The changes in mRNAs encoding uPA and fibronectin mirror the changes observed in the synthesis and secretion of these proteins.

Effects of 8-Bromo-cAMP on uPA mRNA Levels and uPA Secretion—Exposure of cytotrophoblasts to 1.5 mM 8-bromo-cAMP promoted a 3-fold increase in uPA mRNA (3.2 ± 0.6-fold, X ± S.E., n = 3) and secreted uPA activity during the first 24 h of culture (Figs. 1 and 3). However, this stimulation was transient as uPA mRNA and secreted uPA activity declined during the subsequent 24 h of incubation. 8-Bromo-cAMP treatment reduced expression of fibronectin mRNA, but markedly increased the levels of the 1.05-kilobase mRNA encoding β-hCG at 24 and 48 h of treatment. In addition, the secretion of hCG into the medium per day was increased by more than 10-fold compared to controls during the first 24 h of treatment and by more than 50-fold after 48 h. (Values are X ± S.E., n = 3; control, 24 h, 10 ± 1.7 mIU/ml; 8-bromo-cAMP, 24 h, 143 ± 29 mIU/ml; control, 48 h, 25 ± 8 mIU/ml; 8-bromo-cAMP, 48 h, 1398 ± 163 mIU/ml.) Therefore, the cAMP-stimulated expression of endocrine function of the trophoblasts is sustained (5, 6). Actin mRNA levels, determined by probing of the filters with a β-actin cDNA (18), declined ~80% after 48 h of culture. 8-bromo-cAMP caused a 65% reduction in actin message at 24 and 48 h.

DISCUSSION

Human trophoblast has been shown to degrade extracellular matrix (19). The present study reveals for the first time that human cytotrophoblasts synthesize and secrete uPA, which may well account, in part, for the previously described capacity of placental cells to break down extracellular matrix proteins. It is intriguing that the production of uPA by cultured trophoblasts is evanescent. Activity is expressed primarily during the first 24 h of culture and then declines. The fall in uPA occurs in concert with a rise in the level of mRNA encoding the extracellular matrix protein, fibronectin, under our basal culture conditions. This temporal pattern of initial
production of a protease which degrades extracellular matrix followed by increased elaboration of an extracellular matrix protein raises the possibility that these processes might be coupled in some way.

The factors which stimulate uPA synthesis and secretion by trophoblasts and then suppress its expression remain to be determined. cAMP seems to have some role in the up-regulation of uPA in trophoblasts as it has in other cell systems (3). Preliminary studies have revealed that uPA mRNA levels in the cytotrophoblasts are increased within the initial 4 h after addition of 8-bromo-cAMP, demonstrating a relatively rapid response. However, the stimulatory effect of the cAMP analog is short lived, as uPA mRNA levels are markedly reduced after 48 h of culture in the presence of the cyclic nucleotide. The inability of 8-bromo-CAMP to sustain trophoblast uPA secretion is not due to development of a refractory state since β-hCG mRNA and hCG secretion are increased throughout the exposure to the cyclic nucleotide analog (5, 6). The factors responsible for the circumscribed stimulation of plasminogen activator expression in these systems remain to be explored.

Acknowledgments—We thank Alice Kuo and Susan Murray and Drs. Elliot Barnathan and Aaron Hsueh for their assistance in various aspects of these studies. We are grateful to Barbara Brewer for help in preparing this manuscript.

REFERENCES
1. Blasi, F., Vassalli, J.-D., and Dano, K. (1987) J. Cell Biol. 104, 801–804
2. Strickland, S., Reich, E., and Sherman, M. I. (1976) Cell 9, 231–240
3. Nagamine, Y., Sudol, M., and Reich, E. (1983) Cell 32, 1181–1190
4. Kliman, H. J., Nestler, J. E., Sermasi, E., Sanger, J. M., and Strauss, J. F., III (1986) Endocrinology 118, 1567–1582
5. Feinman, M. A., Kliman, H. J., Calitabiano, S., and Strauss, J. F., III (1986) J. Clin. Endocrinol. Metab. 63, 1211–1217
6. Ulloa-Aguirre, A., August, A. M., Golos, T. G., Kao, L.-C., Sakuragi, N., Kliman, H. J., and Strauss, J. F., III (1987) J. Clin. Endocrinol. Metab. 64, 1002–1009
7. Granelli-Piperno, A., and Reich, E. (1978) J. Exp. Med. 148, 223–234
8. Heussen, C., and Dowdle, E. B. (1980) Anal. Biochem. 102, 196–202
9. Laemmli, U. K. (1970) Nature 227, 680–685
10. Ivarie, R. D., and Jones, P. P. (1979) Anal. Biochem. 97, 24–35
11. Kessler, S. W. (1981) Methods Enzymol. 73B, 442–459
12. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Berent, S. L., Mohmoudi, M., Torczynski, R. M., Bragg, P. W., and Bollon, A. P. (1985) Biotechniques 3, 208–220
14. Verde, P., Stopelli, M. P., Galeffi, P., Di Nocera, P., and Blasi, F. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4727–4731
15. Fiddes, J. C., and Goodman, H. M. (1980) Nature 286, 684–687
16. Bernard, M. P., Kolbe, M., Weil, D., and Chu, M.-L. (1985) Biochemistry 24, 2698–2704
17. Ny, T., Bjersing, L., Hsieh, A. J. W., and Loskutoff, D. J. (1985) Endocrinology 116, 1666–1668
18. Gunning, P., Fonte, P., Okayama, H., Engel, J., Blau, H., and Redes, L. (1983) Mol. Cell. Biol. 3, 787–795
19. Fisher, S. J., Leitch, M. S., Kantor, M. S., Basbaum, C. B., and Kramer, R. H. (1985) J. Cell Biochem. 27, 31–41
20. O’Connell, M. L., Canipari, R., and Strickland, S. (1987) J. Biol. Chem. 262, 2339–2344