JOURNAL OF VIROLOGY, Aug. 1979, p. 415–419
0022-538X/79/08-0415/05$02.00/0

Vol. 31, No. 2

Production of Plasminogen Activator by Human and Hamster Cells Infected with Human Cytomegalovirus

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Received for publication 4 December 1978

Plasminogen activator was produced by both human embryo fibroblasts (a permissive system) and hamster embryo fibroblasts (a nonpermissive system) after exposure to human cytomegalovirus. The level of this activator was measured by using plates coated with [^{125}I]fibrin. The production of plasminogen activator was enhanced when the human cells were exposed to human cytomegalovirus previously irradiated with UV light (5,520 to 55,200 ergs/mm^2).

When human fibroblast cells are infected with human cytomegalovirus (HCMV), host cell DNA (7, 20), RNA (21), proteins (19), DNA polymerase (9, 11), thymidine kinase (5, 26), and ornithine decarboxylase (13) are stimulated. These characteristics are common to oncogenic DNA viruses. In addition, HCMV has recently been shown to transform human embryo fibroblast (8) and hamster embryo fibroblast (HEF; 2) cells to a cancerous state. Therefore, it is of some interest to establish further properties of this common human pathogen that may relate to malignancy.

A variety of biochemical differences between normal and transformed cells have been reported, including release of a protease, plasminogen activator by transformed cells (3, 22). This enzyme converts a serum plasminogen to plasmin, which in turn hydrolyzes fibrin. Unkeless et al. (23) and Ossowski et al. (16) reported that transformation of cells by oncogenic RNA and DNA viruses is associated with the appearance of greatly increased levels of plasminogen activator. This report describes the production of plasminogen activator by cells infected with HCMV.

MATERIALS AND METHODS

Cell culture and virus. Whole human embryo cells (Flow 5000), purchased commercially from Flow Laboratories, Inc., Rockville, Md., were subcultured at 6- to 7-day intervals, using Dulbecco medium supplemented with 10% fetal calf serum, 0.075% $NaHCO_3$, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The methods used for preparation of HEF cells were, in general, those described previously by Duff and Rapp (4).

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HCMV strains AD169 and ET were obtained from S. C. St. Jeor, and strain Mj was obtained from L. Geder in this laboratory. Preparation of virus stocks has been described previously (25). HCMV titers were measured in Flow 5000 cells by the plaque method of Wentworth and French (24). Tests for mycoplasma (17) were negative.

Infection procedure. Flow 5000 and HEF cells were subcultured in 60-mm plastic plates (Corning Glass). Virus solution (0.5 ml) was inoculated onto confluent cell cultures and adsorbed for 1 h at 37°C. After the infected cultures were washed with phosphate-buffered saline, Dulbecco medium supplemented with 1% heat-inactivated (56°C, 30 min) fetal calf serum was added, and the cultures were incubated at 37°C. Medium was then changed to Dulbecco medium supplemented with 2.5% dog serum.

UV irradiation. Two-milliliter samples of virus stock were irradiated in 60-mm plastic plates with a UV-germicidal lamp (General Electric G8T5) at a distance producing a radiation dose of 42 ergs/s per mm^2.

Determination of plasminogen activator. Bovine fibrinogen (94% clottable) (Sigma Chemical Co., St. Louis, Mo.) was iodinated by using chloramine-T as an oxidizing agent. To the radioiodinate, 0.2 ml of fibrinogen (5 to 10 mg), 1 μCi of [^{125}I]Na (New England Nuclear Corp., Boston, Mass.), and 0.2 ml of freshly prepared chloramine-T (2 mg/ml) were added in a small vial, and the mixture was shaken occasionally for 5 min at room temperature. The reaction was stopped by the addition of 0.2 ml of Na_2SO_3 (2.4 mg/ml) and 0.04 ml of 1 M KI. [^{125}I]fibrinogen was separated from the reaction mixture by chromatography on a 7-ml Sephadex GT-25-80 column. Before use, the column was equilibrated with 2 column-volumes of TEN (20 mM Tris-hydrochloride [pH 7.6], 1 mM EDTA, 100 mM NaCl) buffer, loaded with 0.5 ml of 20 mg of bovine serum albumin per ml in TEN buffer, and washed with 2 column-volumes of TEN supplemented with 1 mg of bovine serum albumin per ml. The elution buffer was TEN supplemented with 1 mg of bovine serum albumin per ml. Samples of the fractions were precipitated with trichloroacetic acid and

counted. ^{125}I-labeled fibrin-coated dishes (35-mm plastic plates) were prepared as described by Unkeless et al. (23). Dishes were prepared, each containing [^{125}I]-fibrinogen (10 $\mu g/cm^2$; total radioactivity, 1.2×10^5 cpm).

The infected or mock-infected cultures were washed with phosphate-buffered saline, and Dulbecco medium containing 2.5% dog serum was added to the cultures. Cultures were incubated for 12 h at 37°C. After collection of culture fluid, the fluid was frozen at −80°C until tested. The fibrinolytic reaction was performed with 1.5 ml of harvest fluid per plate and incubated for 5 h at 37°C. The radioactivity released into the medium was followed by removing the fluid and measuring the radioactivity in a gamma counter (Beckman). Plasminogen-depleted dog serum was kindly supplied by M. K. Howett in this laboratory.

RESULTS

Production of fibrinolytic activity by HCMV in a virus-permissive system. Confluent Flow 5000 cells were infected with HCMV (AD169) at a multiplicity of infection (MOI) of 5, 0.5, or 0.05 PFU/cell. The medium was changed to Dulbecco containing 2.5% dog serum at day 2, 3, 4, or 7. The method of fibrinolytic activity assay was described in Materials and Methods.

Cultures of mock-infected cells did not exhibit fibrinolytic activity (less than 2%) (Table 1). When cells were infected with AD169 at an MOI of 5 PFU/cell, 6% of the radioactivity of total insoluble [^{125}I]fibrin was released into the fluid 2 days postinfection (p.i.), and the released radioactivity decreased 3, 4, and 7 days p.i. In cultures infected with AD169 at an MOI of 0.5 PFU/cell, the radioactivity released was 40.9 and 50.3% at days 2 and 3, respectively. However, there was no increase in fibrinolytic activity in cultures infected at an MOI of 0.05 PFU/cell. Similar results were obtained with the ET strain.

Plasminogen depletion of dog serum. The experiments in Table 2 show the plasminogen dependence of this process. When plasminogen-depleted dog serum was used for this assay, the radioactivity released into the fluid was lower (2.2%) than that of the mock-infected cultures (3.5%). When fetal calf serum containing low plasminogen (15) was applied to this system, the radioactivity released was lower than when dog serum was used.

Ability of UV-irradiated HCMV to induce plasminogen activator. The effect of various doses of UV irradiation of the virus on the induction of plasminogen activator was tested. The titers of UV-irradiated virus decreased, and the inactivation rate corresponded to the UV dose applied (Fig. 1). However, high levels of plasminogen activator were observed even when HCMV was irradiated up to 55,200 ergs/mm^2 (Table 3). The highest production of plasminogen activator was detected when the UV dose was 5,520 ergs/mm^2, and the radioactivity released into the medium was 82.7% at day 1. When HCMV was irradiated with a dose of 151,200 ergs/mm^2, no plaques were formed even when the cell cultures were infected with undiluted virus, and no significant quantity of plasminogen activator was produced by the cells.

Production of plasminogen activator in a nonpermissive system. Although HCMV can express some gene functions in HEF cells, it does not replicate in these cells (1). HEF cell cultures were infected with HCMV strain AD169, ET, or Mj. When HEF cells were exposed to nonirradiated AD169 at an MOI of 0.5 PFU/cell, the radioactivity released into the medium was 10.8

TABLE 1. *Fibrinolytic activity in Flow 5000 cells infected with HCMV*[a]

Days p.i.	Radioactivity released into solution (% of total)			
	Mock infected	AD169		
		MOI 0.05	MOI 0.5	MOI 5
2	2.1	1.6	40.9	6.0
3	1.1	2.6	50.3	2.0
4	1.6	ND[b]	ND	1.7
7	1.2	1.1	1.2	1.2

[a] Plates (35-mm diameter) containing [^{125}I]fibrin (10 $\mu g/cm^2$; total radioactivity, 1.0×10^5 cpm) were prepared as described in the text. Media in cultures infected with mock or HCMV were changed to dog serum (2.5%)-containing media and incubated for 12 h at 37°C. Incubation in [^{125}I]fibrin-coated plates was carried out for 5 h at 37°C.

[b] ND, Not done.

TABLE 2. *Effect of plasminogen activator-depleted serum or fetal calf serum on fibrinolytic activity of Flow 5000 cells infected with HCMV*[a]

Serum	Radioactivity, cpm, released into solution (% of total)	
	Mock infected	HCMV infected
2.5% dog serum	3,500 (3.5)	52,800 (52.8)
2.5% plasminogen-depleted dog serum	900 (0.9)	2,100 (2.2)
2.5% fetal calf serum	1,400 (1.4)	2,680 (2.7)

[a] Plates (35-mm diameter) containing [^{125}I]fibrin (10 $\mu g/cm^2$; total radioactivity, 1.0×10^5 cpm) were prepared as described in the text. Flow 5000 cells were infected with HCMV (AD169) at an MOI of 0.5 PFU/cell, and medium was changed to dog serum (2.5%)- or fetal calf serum (2.5%)-containing medium 3 days after infection. Incubation was continued for 12 h at 37°C. Incubation time for the plasminogen activator in [^{125}I]fibrin-coated plates was 5 h at 37°C.

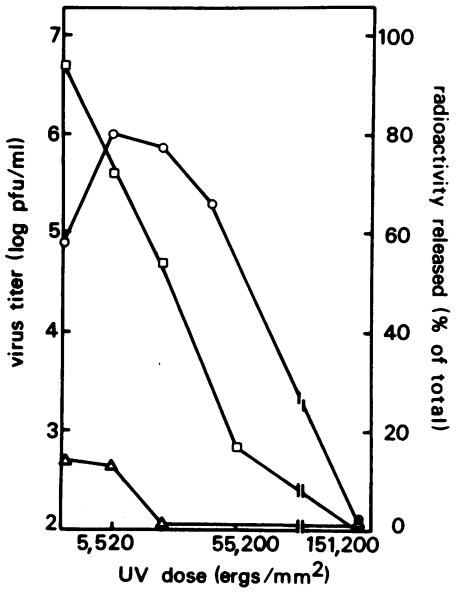

FIG. 1. *Effect of UV irradiation of HCMV on the production of plasminogen activator in Flow 5000 and HEF cells. Confluent Flow 5000 and HEF cells were exposed to nonirradiated or UV-irradiated HCMV at an MOI of 0.5 PFU/cell (for nonirradiated virus). The plasminogen activator assay was carried out as described in the text. Symbols: (□) infectivity; (○) radioactivity released into medium (percentage of total) from Flow 5000 cells; (△) radioactivity released into medium (percentage of total) from HEF cells.*

and 13.5% at days 2 and 3, respectively (Table 4). Similar results were obtained in cultures infected with UV-irradiated (5,520 ergs/mm^2) virus. However, in contrast to the Flow 5000 cell cultures infected with UV-irradiated virus (13,800 ergs/mm^2), there was no production of plasminogen activator by more heavily irradiated virus. Similar results were obtained with 5 PFU and with the ET and Mj isolates of HCMV.

DISCUSSION

The results obtained demonstrate that plasminogen activator is produced in virus-permissive (Flow 5000) and nonpermissive (HEF) cells exposed to HCMV. The production of plasminogen activator reached a peak 3 days after infection when the Flow 5000 cell cultures were infected with nonirradiated HCMV AD169 at an MOI of 0.5 PFU/cell. However, when cells were infected at an MOI of 5 PFU/cell, the production of plasminogen activator was lower than when they were infected at an MOI of 0.5 PFU/cell and decreased to the mock-infected level 3 days after infection (Table 1). When human embryo fibroblast cells are infected with HCMV, cell DNA synthesis and a variety of cell enzymes (5, 11, 13, 20) are stimulated, and stimulation of these functions appears to be multiplicity dependent. However, the production of plasminogen activator was not enhanced when cell cultures were infected with HCMV at a high MOI. When human fibroblast cells were infected with a high MOI of HCMV, early cell rounding was observed (6). The factor(s) responsible for early cytopathology may be involved in the inhibition of plasminogen activator production by high multiplicities of HCMV.

When cultures are infected with HCMV, infectious HCMV is synthesized between 2 and 3 days p.i. and HCMV DNA is also detectable approximately 48 h p.i. (9, 20). Stimulation of cellular thymidine kinase is seen within 12 h (5),

TABLE 3. *Fibrinolytic activity in Flow 5000 cells infected with UV-irradiated HCMV*[a]

Irradiation dose (ergs/mm^2)	Radioactivity released into solution (% of total) at days p.i.:			
	1	2	4	6
0	44.9	58.3	11.3	3.2
5,520	82.7	80.3	67.0	3.8
13,800	77.8	77.4	70.0	58.0
55,200	76.5	66.6	71.4	10.3
151,200	2.3	2.3	3.5	2.1
Mock infected	1.5	2.4	2.9	2.3

[a] Plates (35-mm diameter) containing [^{125}I]fibrin (10 μg/cm^2; total radioactivity, 1.0 × 10^5 cpm) were prepared as described in the text. Flow 5000 cells were infected with nonirradiated or irradiated HCMV (AD169) at an MOI of 0.5 PFU/cell (titer before irradiation). Medium of infected cultures was changed to dog serum (2.5%)-containing medium. Incubation was continued for 12 h at 37°C. Incubation time for the plasminogen activator in [^{125}I]fibrin-coated plates was 5 h at 37°C.

TABLE 4. *Fibrinolytic activity of HEF cells exposed to HCMV strain AD169*[a]

UV irradiation dose (ergs/mm^2)	Radioactivity released into solution (% of total) at days p.i.:		
	2	3	7
0	10.8	13.5	0.9
5,520	9.0	13.1	1.0
13,800	2.5	2.2	0.9
151,200	2.3	1.6	1.1

[a] Plates (35-mm diameter) containing [^{125}I]fibrin (10 μg/cm^2; total radioactivity, 1.0 × 10^5 cpm) were prepared as described in the text. HEF cells were infected with HCMV. Medium of infected cultures was changed to dog serum (2.5%)-containing medium. Incubation was continued for 12 h at 37°C. Incubation time for the plasminogen activator in [^{125}I]fibrin-coated plates was 5 h at 37°C. The MOI was 0.5 PFU/cell.

induction of cell DNA polymerase occurs at 20 h p.i. (9), stimulation of ornithine decarboxylase activity is apparent as early as 12 h p.i. (13), and the production of plasminogen activator is detected 24 h p.i. The production of plasminogen activator does not require virus DNA replication. Plasminogen activator was produced in HEF cells in which virus DNA (data not shown) and infectious virions were not synthesized, indicating that production of plasminogen activator is an early event (Table 4).

UV irradiation of HCMV enhanced the production of plasminogen activator (Table 3). Hirai et al. (10) reported that irradiation of HCMV at a low UV irradiation dose resulted in an enhancement of cell DNA synthesis, transformation, and simian virus 40 T-antigen induction (18), events that may be analogous to the enhancement of plasminogen activator.

Even if highly irradiated (13,800 to 55,200 ergs/mm^2) viruses were used in these tests, the production of plasminogen activator remained at high levels in Flow 5000 cell cultures (Table 3). The resistance of HCMV to UV irradiation also was reported during induction of cell DNA synthesis by HCMV (10). However, the mechanism for this is unknown.

There is convincing evidence that malignant cells produce plasminogen activator (3, 16, 23) and that the production appears to be closely related to malignant transformation (16, 23). HCMV can induce oncogenic transformation of hamster (2) and human embryo fibroblast (8) cells. These transformed cells may produce plasminogen activator (12; Yamanishi, unpublished data). When human diploid fibroblasts were inoculated with highly diluted HCMV or UV-irradiated HCMV, they grew in soft agarose for several generations before undergoing lysis due to virus cytopathology (14). Albrecht et al. (1) reported that HCMV is capable of inducing mitosis in hamster cells. Our results indicate that HCMV is able to induce synthesis of plasminogen activator in permissive and nonpermissive cells. However, this production did not persist, presumably due to the cytopathic effects of the virus. These data imply that HCMV has the capacity to transform cells and that abortive transformation may occur in cells subsequently destroyed by the virus.

ACKNOWLEDGMENTS

We thank Nancy Turner and Sara Fetrow for technical assistance.

This investigation was supported by Public Health Service contract N01 CP 53516 within the Virus Cancer Program of the National Cancer Institute and grant CA 18450 awarded by the National Cancer Institute.

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