FIBRINOLYTIC ACTIVITY OF ADULT RAT LIVER CELLS IN PRIMARY CULTURE AND INHIBITION BY GLUCOCORTICOIDS

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SUMMARY

Adult rat liver cells, maintained in primary culture in a non-proliferating state, have been observed to flatten and spread-out during the first 3 days in culture. A fibrin-agar overlay technique and the digestion of ^{125}I-fibrin films have been used to show the potent fibrinolytic activity of liver cells in primary culture. Microscopic observation of liver cells under fibrin-agar overlays demonstrated the potent fibrinolytic activity of individual flattened liver cells. Both the fibrinolytic activity and the flattening of the liver cells are inhibited by dexamethasone and to a lesser degree by hydrocortisone. Fibrin digestion per se is unaffected by dexamethasone supplementation.

INTRODUCTION

Functional adult-rat hepatocytes, when attached to polystyrene culture vessels, have been observed to change from an initial, rounded ("epithelial-like") shape to a flat, spread-out morphology over a culture period of 3 days. Dexamethasone (dex) delayed the flattening of the cell shape (1,2). Neither the mechanism of the morphologic alteration from round to flat, nor the dexamethasone inhibition of the cell-spreading phenomenon are understood. In view of the suggested relationship between proteolytic activity and cell morphology (see 9), it became of interest to investigate whether the changes in shape of isolated liver cells in culture could be related to a similar phenomenon.

These considerations led to the findings that, in primary culture, isolated rat liver cells from normal animals show active fibrinolytic activity and that glucocorticoids inhibit both this activity and cell

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Abbreviations used: PI: post-inoculation, refering to the time elapsed from the inoculation of dissociated liver cells into the flask; dex: dexamethasone; HC: hydrocortisone; FBS: fetal bovine serum; CFS: cell-free supernatant; WE: Williams' Medium E.

spreading. A well-known malignant neoplasm of liver cells, the HTC cell line (8) produces almost no fibrinolysis under similar conditions.

MATERIALS AND METHODS

Animals. Adult, male Fischer-344 albino rats (Charles River) weighing 250-320g were used. Animals were fed Purina lab chow and water ad libitum and maintained on a 12-hr light cycle.

Hormones. Dexamethasone and hydrocortisone were purchased from Sigma Chemical Co., St. Louis, Mo. Stock solutions were prepared in 70% ethanol (e.g. 19.65 mg dex in 2.5 ml 70% ethanol) every 7 - 10 days and kept at 4°. Control cultures received equivalent volumes of 70% ethanol.

Liver Cell Cultures. All cell preparations were made between 900 and 1200 hr. The dissociation of adult rat liver and handling of the resulting cell preparations were done as previously described (1) with two modifications: (a) the concentration of collagenase (Sigma Type I, Lot #25C-0289) was increased to 0.05%, and (b) the rate of collagenase perfusion was decreased to 7 ml/min during the final 60 sec of perfusion. In this manner, viabilities of 80 - 90% (as estimated by rigorously controlled trypan blue exclusion) (1) were routinely obtained.

Plaque-Assay of Fibrinolysis. Each culture flask (Falcon #3013, 25 cm^2 surface area) was inoculated with 10^5 viable cells in 4 ml of culture medium (Williams' Medium E, Flow Laboratories) (WE), containing 10% fetal bovine serum (Flow Lot #40551041) (FBS). All WE used in these experiments contained antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml) and fungizone (amphotericin B 0.25 µg/ml). The cells were allowed to attach in all experiments for 5 hr, following which the attachment medium was removed, the cultures washed (x2) with WE and replenished with 3 ml of WE plus FBS (10%). To ascertain the number of attached viable cells, all floating debris was removed, the cells were detached with 0.25% trypsin, and counted in a hemacytometer chamber under defined conditions with trypan blue (1).

At the time of overlay, the cultures were washed once with serum-free WE. Bovine thrombin (Parke-Davis, Lot #908251C, 5 units in 50 µl WE) was added to each flask followed by a pre-mixed solution (40°) containing bovine fibrinogen (Sigma Type I, Lot #115C-0162, 0.6 ml, 5 mg/ml in WE), FBS (0.1 ml), and agar (0.3 ml, 20 mg/ml in WE). Difco purified agar was dissolved, boiled 20 min prior to use, and maintained at 42° during overlay procedure. The overlay solution was added rapidly to the flask, mixed rapidly with the thrombin in the flask and distributed uniformly over the cell layer. The agar hardened within 20 sec. The flasks were maintained at 37° in a water-saturated atmosphere of 5% CO_2 in air. The overlay became opaque as the fibrinogen clotted in 5 - 10 min (3 - 6).

Plaque formation in the fibrin-agar overlay could be monitored without fixation or staining by illuminating the overlay from any side. For preservation, the cultures were fixed and stained with Coomassie blue in methanol/acetic acid/water for 2 hr (3). Lysis zones were scored macroscopically and visualized best by wetting the surface of the dried, stained overlay with water. Fibrinolytic activity was determined in most experiments during the first 3 days of non-proliferating culture to avoid possible interference by the few foci of proliferating cells appearing after 3 days PI.

Photomicrography. Cultures with fibrin-agar overlays were flooded with the Coomassie blue fixation solution (3) for 15 - 30 min, drained, rinsed well with water and photographed when wet on a Nikon Inverted

Phase-Contrast Microscope using Kodak Contrast Process Ortho film. The film was developed in HC-110 (Kodak).

^{125}I-Fibrin Plate Assay of Fibrinolysis. The ^{125}I-Fibrin-coated culture dishes were prepared from purified bovine fibrinogen as described by others (3 - 6). For the collection of cell-free supernatants, 25 cm^2 flasks were inoculated with 5 - 10 x 10^5 viable cells, washed after 5 hr, and maintained in WE (10% FBS). At the beginning of the collection period, the cultures were washed (x3) with WE, fed with 1 ml WE, gassed with CO_2 to control pH, closed, and rocked for 11 hr on a layer of water (37°) in a shaker bath (120 strokes/min). The media were collected and cleared of debris by centrifugation (1000 x g, 10 min). Aliquots were diluted with serum-free WE and added to the ^{125}I-fibrin plates with and without FBS for 18 hr (7). The ^{125}I in 1 ml of the clear digest fluids was counted on an Intertechnique Gamma Spectrometer, Model CG30.

RESULTS

Detection of fibrinolytic activity with the fibrin-agar overlay method permitted a direct microscopic comparison between the morphology of the liver cells and the appearance of fibrinolysis zones.

To ascertain the relationship between the number of attached viable cells and the number of lysis zones generated per flask, various numbers of viable cells from a fresh dissociate were analysed for fibrinolytic activity over a 24 hr period. A count of the number of lysis zones per flask compared with a determination of the attached viable cells in duplicate flasks at the time of overlay, revealed an increase in numbers of lysis zones with increased numbers of attached viable cells (Table 1).

Supplementation of the culture medium and the overlay medium with

Table 1. Relationship between the number of fibrinolysis zones in culture flasks containing different numbers of attached, viable, rat liver cells.

(1) Number of Attached Viable Cells[a]	(2) Number of Lysis Zones per Flask (±S.E.)[b]	Ratio $\frac{(2)}{(1)} \times 10^{-3}$
0.04 x 10^5	94 ± 5	2.4
0.14 x 10^5	372 ± 21	2.7
0.52 x 10^5	1200[c]	2.3

a. Average of counts from two flasks determined at 16 hr PI
b. Overlay duration: 24 hr (16 - 40 hr PI). Mean of determinations from 3 flasks.
c. An estimate. Some zones overlap extensively.

Table 2. Effects of Dexamethasone Concentration and of Hydrocortisone on the Production of Fibrinolysis Zones.

	Number of Lysis Zones Per Flask (±S.E.)[a]		
Hormone Additive	8-48 hr PI Overlay	8-72 hr PI Overlay	Extent of Cell Spreading at 72 hr[c]
Control (No Hormone)	800-1000[b]	1200[b]	++++
Dex (10^{-6}M)	0	0	+
Dex (10^{-8}M)	67±10	128±20	++
HC (10^{-4}M)	0	5.4±1.7	++

a. Mean of determinations from 3 flasks.
b. An estimate. Some zones overlap extensively.
c. Evaluated with or without a fibrin-agar overlay.
+: Some cells (about 5-10%) are spreading slightly
++: About 20-60% of the cells are spreading to various degrees.
++++: About 95% of the cells are spreading extensively.

dex or HC revealed a marked inhibition of the production of lysis zones (Table 2). In the experiment illustrated, complete inhibition of the production of lysis zones was seen at 48 hr and 72 hr PI with 10^{-6}M dex whereas partial inhibition with 10^{-8}M dex or 10^{-4}M HC was observed at 72 hr PI. Microscopic evaluation of the extent of cell spreading at 72 hr PI revealed that 10^{-6}M dex was most effective in inhibiting the time-dependent spread of the liver cells, whereas 10^{-8}M dex and 10^{-4}M HC were moderately effective.

In addition, it was observed microscopically that virtually every lysis zone contained a spread-out cell (Figure 1). It is to be noted that the scoring of lysis zones was done macroscopically although microscopic lysis zones (each containing a spread-out cell) could be observed. Interestingly, the use of the fibrin-agar overlay allowed degenerating cells (rounded and fragmented) to be contained and localized and these were observed to produce no macroscopic zones of fibrinolysis (Figure 1).

The reversibility of the 10^{-6}M dex inhibition of fibrinolysis zone formation was evaluated by maintaining cultures for 16 hr PI in 10^{-6}M dex medium and then overlaying the cultures with fibrin-agar. The overlays

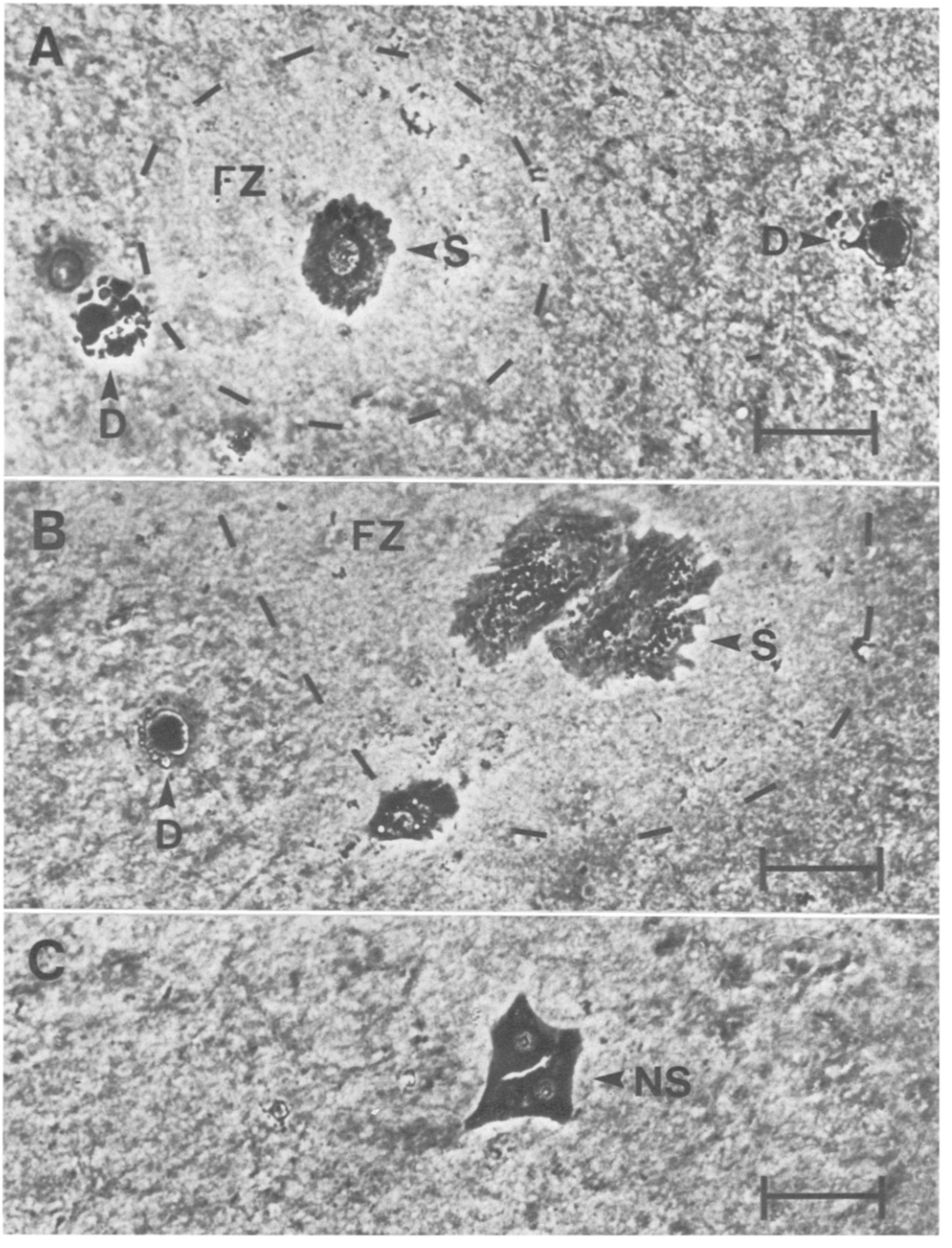

Figure 1. Photomicrograph of Cultured Adult Rat Liver Cells Maintained under a Fibrin-Agar Overlay from 16 to 88 hr PI (72 hr). Cells were exposed to control medium only. A: Cell with extended cytoplasm is a spread-out cell(S) and lies within a clear, less granular area characteristic of large fibrinolysis zones (FZ) after fixation and staining (see text for method). Two degenerating cells (D) are not surrounded by FZ. B: Degenerating cell (D) lies outside FZ surrounding a doublet of spread-out cells(S). The cell at the edge of FZ appears only slightly spread-out. C: The doublet of cells exhibiting few cytoplasmic extensions is non-spread (NS) and is not contained within a fibrinolysis zone. Few such NS cells are found at 88 hr PI in control medium. The horizontal bars represent 40μ.

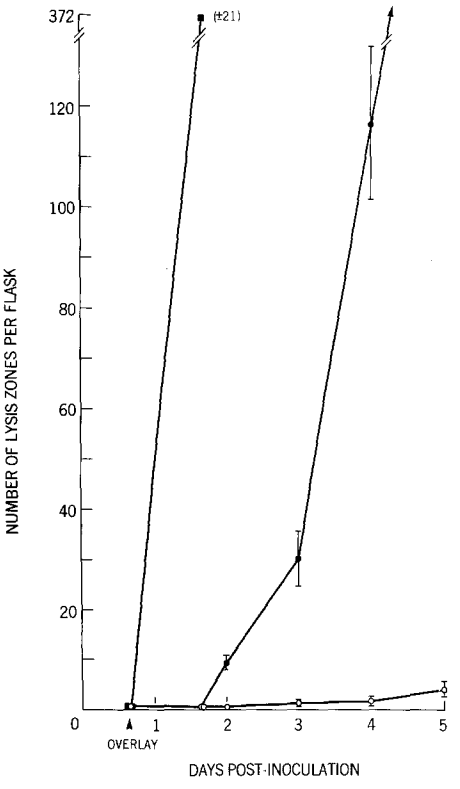

Figure 2. Reversibility of Dexamethasone Inhibition of Fibrinolysis Zone Formation. Cell cultures established in: control medium with control medium in overlay (■—■), 10^{-6}M dex medium with control medium in overlay (●—●), 10^{-6}M dex medium with 10^{-6}M dex medium in overlay (O—O).

contained either control medium or 10^{-6}M dex medium. The results clearly show that the inhibition of both fibrinolysis (and cell spreading) requires the continued presence of 10^{-6}M dex in the overlay (Figure 2).

To investigate the possibility that viable liver cells that have flattened and spread-out on polystyrene are capable of producing measurable fibrinolytic activity sooner than cells that are more rounded, cultures established in control medium or 10^{-6}M dex medium were subjected to short "pulse" overlays at 1, 2, and 3 days PI. Cultures established in control medium received control medium in the overlays, while cultures established in 10^{-6}M dex medium received 10^{-6}M dex medium in the overlays. To account for variabilities in numbers of attached viable cells, cell counts were conducted in duplicate flasks at the times of overlay and the numbers of fibrinolysis zones were expressed in terms of 10^{5} attached viable cells.

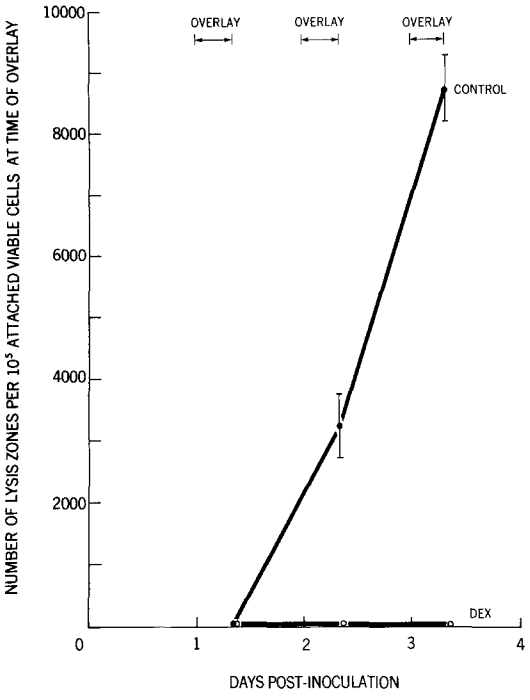

Figure 3. Relationship of the Rate of Formation of Fibrinolysis Zones to the Length of the Culture Period before Overlay. Cell cultures established in: control medium with control medium in the overlay (●—●); 10^{-6}M dex medium with 10^{-6}M dex medium in the overlay (○—○). The duration of the 8-hr overlay periods is indicated at the top of the graph.

It is clear that the fibrinolysis zone formation in control cultures is much more rapid at the later overlay periods (2 and 3 days PI) when cells are spread-out to a greater extent (Figure 3). The inhibitory effect of 10^{-6}M dex is complete in all periods. The results indicate that about 1 out of 10 attached viable cells in control cultures is capable of producing a macroscopic fibrinolysis zone within an 8-hr overlay period at 72 hr PI, under the conditions described.

Analysis of the fibrinolytic activity of the rat hepatoma cell line, HTC (8), by the fibrin-agar overlay technique (3-day overlay duration) revealed only a trace of activity (about 1 fibrinolysis zone per 10^5 attached viable HTC cells) as did primary cultures of isolated liver macrophages (data not shown).

The spread cells within the lysis zones were viable. This was assessed by first marking the location of lysis zones, removing the unfixed fibrin-agar overlay, and then adding trypan blue (1) directly to the culture flask.

Table 3. Fibrinolytic activity of cell-free supernatants (CFS) measured by the extent of solubilization of bovine ^{125}I-fibrin films.

Collection Period	Cell Densities (per Flask)	Supplement[a]	Amount of CFS[b] (%)	Amount of FBS[c] (%)	Fibrin Solubilized[d] (%)
72-83 hr PI	0.5-1.5x10^5	Control	1.0	0	6.1
			2.5	0	25
			5.0	0	54
			10	0	78
			20	0	85
			10(10^{-6}M Dex)[e]	0	78
			10(10^{-5}M Dex)[e]	0	79
24-35 hr PI	5-7x10^5	Control	10	0	60
			10	10	65
	5-7x10^5	Dex	10	0	0
			10	10	0
48-59 hr PI	3-5x10^5	Control	10	0	82
			10	10	85
	4-6x10^5	Dex	10	0	0.02
			10	10	0.03
72-83 hr PI	0.5-1.5x10^5	Control	10	0	77
			10	10	89
	3-5x10^5	Dex	10	0	0.19
			10	10	0.15

a. Either control medium or dex (10^{-6}M) supplementation of both culture medium and serum-free supernatant.
b. CFS diluted with WE to give total volume of 1 ml.
c. Fresh FBS added to CFS+WE immediately prior to addition to ^{125}I-fibrin plate: Total volume: 1 ml.
d. Values are expressed as a percent of the ^{125}I-fibrin digested by 0.25% trypsin in WE during the 18 hr incubation period. Values are corrected for the radioactivity released (less than 2%) by WE alone or WE + FBS.
e. Dex was added to the CFS immediately prior to ^{125}I-fibrin digestion. The final dex concentration on the overlay is indicated in brackets.

The solubilization of ^{125}I-fibrin by CFS collected from cultures established in control medium or 10^{-6}M dex medium corroborate the observation that 10^{-6}M dex inhibited the production of fibrinolysis zones produced at 1, 2, or 3 days PI (Table 3). CFS (10%) from control cultures (72 - 83 hr collection period) was found to produce high levels of fibrinolysis (77 - 78%) whereas CFS (10%) from 10^{-6}M dex cultures (72 - 83 hr collection period) produced only trace levels of fibrinolysis (0.19%). Interestingly, the addition of fresh FBS to the diluted CFS appeared to

slightly enhance the fibrinolytic activity in most cases. Dexamethasone did not inhibit the digestion of ^{125}I-fibrin by CFS (Table 3).

DISCUSSION

This study demonstrates, for the first time, the potent fibrinolytic activity of liver cells, from adult rats, maintained as primary, non-proliferating monolayer cultures. In view of the recent interest in establishing the role of fibrinolytic activity in malignant cell transformation (4 - 6), it is interesting that the fibrinolytic activity observed in the primary liver cell cultures from normal rats was far greater than that observed with monolayer cultures of the malignant rat hepatoma cell line, HTC.

The demonstrated correlation between fibrinolytic activity and the spreading of rat liver cells on polystyrene suggests that the fibrinolytic protease(s) may play a role in the dramatic change in cell morphology. It has recently been shown that exogenous proteases can reversibly rearrange the actin cable network of cultured rat embryo cells (9).

The dramatic control of the production of fibrinolytic activity by glucocorticoids occurs via an unknown mechanism. Glucocorticoids have been shown to lower the levels of so-called "intracellular plasminogen activator" in HTC cells (10), and to inhibit plasminogen activator production in rat tongue organ culture (11).

The role of serum in the fibrinolytic process described herein is not clear. FBS has been used as a source of inhibitor in ^{125}I-fibrin digestion experiments (5) but does not function as an inhibitor in our experiments. Whether the protease activity demonstrated in this report is plasminogen-dependent is still unknown.

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