Dexamethasone Increases the Synthesis and Secretion of a Partially Active Fibronectin in Rat Hepatoma Cells

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ABSTRACT After treatment with dexamethasone, rat hepatoma-tissue culture cells show a markedly enhanced adhesion to the substratum and increased cell-to-cell interaction. In addition, there is a profound change in the production of secretory glycoproteins. Although the relative synthesis and secretion of a gelatin-binding, fibronectinlike glycoprotein is increased threefold, we do not think this protein is responsible for the improved adhesion properties of the cells because the hepatoma cells do not bind normal fibronectin and because the HTC-produced fibronectin is neither bound by fibroblasts nor has it any affinity for ganglioside-containing phospholipid vesicles. Therefore, these hepatoma cells represent a unique system for studying the regulation of fibronectin synthesis by glucocorticoids. Furthermore, analyses of primary fetal rat hepatocytes have shown that these cells, unlike normal adult hepatocytes, synthesize and secrete fibronectin, which is structurally related to the HTC-cell protein. The comparison of this protein with fibronectin from normal cells will allow a structural characterization of the functional defect in the fibronectin synthesized by transformed cells.

The nature of the support matrix for primary cultures of adult hepatocytes has been found to be an important factor in maintaining the differentiated state of the cells (1, 2). Several components are considered to be essential for providing suitable support conditions such as collagen (2, 3), fibronectin, and laminin (4, 5). Although all these components are integral parts of most basal membranes, only the two former are detectable in significant amounts in the biomatrix of normal-adult liver (6). Due to the surface specialization of hepatocytes (7), only certain portions of the plasma membranes have contact with the extracellular matrix. Excluded are the sinusoidal and bile canicular membranes, which appear to be free of adhesion-promoting proteins. Hepatoma cells have often been used for the biochemical analysis of adhesion and cell-to-cell interaction. These, in contrast to primary hepatocytes, offer the advantage of being a stable and easily manipulable tissue-culture system. The tumor cells, however, poorly approximate the cell-surface domains characteristic of liver cells (8). Rat-hepatoma tissue culture (HTC) cells, as well as other transformed liver-cell lines (9), have proven to be very useful in characterizing several liver-specific membrane phenomena (10). These particular cells, however, have an additional unique feature, which is a glucocorticoid-mediated increase in cell adhesion (11, 12). We provide evidence in this communication that this hormone-effected change in adhesion is independent of fibronectin, despite the concomitant increase in synthesis and secretion of that protein by the HTC cells. Preliminary characterization of the hepatoma fibronectin reveals the lack of some of the binding activities considered specific for fibroblast and plasma fibronectin.

MATERIALS AND METHODS

Cells

A newly cloned cell line of rat hepatoma-tissue culture cells (SR-HTC cells) and WI-38 human fibroblasts were grown in monolayer culture in Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum under an atmosphere of 5% CO_2–95% air. Hormonal treatment of HTC cells consisted of the addition of 1 μM dexamethasone to the culture medium. Primary fetal hepatocytes were obtained by collagenase digestion of livers from 19-d fetuses of Buffalo rats following the procedure of Guguen-Guillouzo et al. (13). The cells, showing 95% viability as measured by trypan-blue dye exclusion, were seeded on collagen-coated 3.5-cm plastic petri dishes (2×10^5 cells in 2 ml) in DME lacking arginine but containing 2 mM L-ornithine, 10% dialyzed fetal calf serum, and 0.1 μM dexamethasone. The selective medium was changed after 2 h and then daily. The cells were used after 96 h in culture. Fetal rat fibroblasts were obtained by digestion of the eviscerated body trunks of the rat fetuses with 0.05% trypsin in PBS containing 0.02% EDTA. The washed cells were plated (3×10^5 cells/10 cm^2) and those adhering to the substratum after 2 h, were maintained in DME containing 10% fetal calf serum. After 4 d, secondary cultures were prepared and used for immunofluorescence studies. For fibronectin-binding studies and for collection of metabolically labeled fibronectin, the cells were cultured in medium containing serum that was first passed through a gelatin-Sepharose column in order to remove endogenous fibronectin.

THE JOURNAL OF CELL BIOLOGY · VOLUME 95 OCTOBER 1982 29-40

Labeling and Isolation of Fibronectin

To obtain radiolabeled medium proteins, confluent cell monolayers were washed three times with serum-free or fibronectin-free medium and incubated for 24–48 h in the same medium (1.3 ml/10 cm^2 monolayer) containing either L-[5,6-^{3}H]leucine (1.8 Ci/mmol), L-[6-^{3}H]fucose (20 Ci/mmol), or L-[^{35}S]-methionine (20 Ci/mmol). The culture media were centrifuged for 5 min at 1,000 *g* and then for 60 min at 200,000 *g*. The supernatant fractions were used directly for separation on two-dimensional gels (14) or for isolation of fibronectin. To label rat-plasma fibronectin, 1 ml of citrated rat plasma was iodinated using 1 mCi ^{125}I and 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril as catalyst (15). The radioactive plasma-proteins were separated from the unincorporated label by passage through a column of Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ). Fibronectin was purified from tissue-culture media or plasma by affinity chromatography on immobilized gelatin (16) following the procedure described by Sekiguchi et al. (17). The amount of protein was determined according to Lowry et al. (18) using albumin as a standard.

Gel Electrophoresis

The separation of medium proteins or fibronectin was carried out by two-dimensional gel electrophoresis (14) using in the second dimension a uniform 7.5 or 10% polyacrylamide gel. To compare proteins separated by two-dimensional gels, the spots were localized by autoradiography, cut out of the dried gel, and subjected to partial proteolytic digestion within a 15% polyacrylamide gel using *Staphylococcus aureus* V8 protease (0.1 μg protease per well, digestion for 30 min at 37°C) (19). The radio-labeled pattern was visualized by fluorography (20).

Immunological Methods

The total mixture of proteins that are secreted by dexamethasone-treated HTC cells into serum-free culture medium, was used for immunization of a rabbit. Six subcutaneous injections, at 2-wk intervals, were carried out: the first was in complete, and the subsequent ones in incomplete Freund's adjuvant, and each contained 1 mg of protein. During the following 3 mo, the animal was bled at 2-wk intervals. The pooled sera were used in this study. To obtain fibronectin-specific antibodies for immunofluorescence studies, the immunoglobulins were collected from the above serum pool by twice precipitating with 40% saturated $(NH_4)_2SO_4$. The immunoglobulins were redissolved in 0.2 M sodium carbonate, pH 9.0 (30 mg/ml), and reacted with fluorescein isothiocyanate (modification: 1.5 mol/mol immunoglobulins) (21). The fluorescent antibodies were then passed through a 1-ml column of Sepharose 4B-CL to which 2 mg of purified rat plasma fibronectin was covalently coupled (16). The bound immunoglobulins were eluted in 0.2 M glycine-HCl, 4 M guanidine HCl, pH 3.0, and then chromatographed on Sephadex G-25 in PBS. For immunofluorescence studies, HTC cells, and fetal rat fibroblasts were grown on glass cover slips placed in 3.5-cm dishes. The cells were washed three times with culture medium and then incubated for 30 min at 4° in 1 ml medium containing 10 μg fluorescent antifibronectin immunoglobulin. The cells were inspected using a Leitz-fluorescence microscope.

To quantify the absolute amount of secreted fibronectin, confluent monolayers (75 cm^2) of HTC cells were incubated for 24 h in 10 ml of fibronectin-free culture medium. The ultracentrifuged media were dialyzed for 24 h against 50 mM NH_4HCO_3 containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and then freeze-dried. The residues were redissolved in 1 ml of PBS containing 1% NP-40, 0.1 mM phenylmethylsulfonyl fluoride. Aliquots of 15 μl of concentrated media or purified fibronectin were analyzed by standard crossed immunoelectrophoresis on 1% agarose gels (10 × 10 cm) in 45 mM Tris, 100 mM glycine, 40 mM barbital buffer, pH 8.5, containing 1% NP-40 (22). The first dimension was run at 5 V/cm for 4 h and the second at 2 V/cm for 22 h into an agarose gel containing 5% (vol/vol) of rabbit antiserum against HTC-cell secretory proteins. The patterns were visualized by staining with Coomassie Brilliant Blue or by fluorography. The fibronectin peak was quantitatively analyzed by measurement of the area under the precipitin line and compared to known quantities of fibronectin. The values are expressed in micrograms of fibronectin secreted per day and per milligram of total cell protein measured at the time of medium collection.

Cell Attachment Assay

The assays were carried out essentially as described by Sekiguchi and Hakomori (23). In brief: plastic tissue culture plates with six wells (3.5-cm diameter; Costar, Data Packaging, Cambridge, MA) were incubated at room temperature with 2 ml of PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1 mM PMSF and 40 μg of fibronectin from either rat plasma or HTC cells. After 4 h, the wells were washed extensively with PBS. Some wells were then incubated for 1 h at room temperature with 2 ml of buffer containing 100 μg of affinity-purified rabbit anti-rat fibronectin (see above). WI-38 fibroblasts and HTC cells were released from monolayers by treatment with trypsin-EDTA and washed once with fibronectin-free culture medium followed three times with PBS. Aliquots of 2 ml of cell suspension (6 × 10^5 WI-38 fibroblasts and 1.5 × 10^6 HTC cells) were added to the test well and incubated for 1 h at 37°C. The adherent cells were photographed using a Nikon inverted phase-contrast microscope.

Binding of Fibronectin to Reconstituted Phospholipid Vesicles

Phospholipids used for reconstitution were purified from rat-liver plasma membrane fractions or [^{32}P]phosphate labeled HTC-cell membranes as described elsewhere (24). Total gangliosides were purified from rat brain as outlined by Saito and Hakomori (25). The gangliosides in the final preparation were separated by thin-layer chromatography and visualized by staining with resorcinol (26). Sealed and unilamellelar phospholipid vesicles were reconstituted by deoxycholate dialysis using 1 mg of phospholipids, alone or combined with 80 μg gangliosides/ml reconstitution mixture (24). The formed vesicles were diluted with 50 mM Tris-HCl, 140 mM NaCl, and 1 mM $CaCl_2$, pH 7.6, (= TBS) and brought to 40% (wt/vol) with solid sucrose resulting in a concentration of 500 μg phospholipid vesicles/ml. Aliquots of 1 ml of vesicle suspension were placed into 12-ml nitrocellulose ultracentrifuge tubes for rotor SW41 (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The vesicles were sequentially overlaid with 0.5 ml of 35% sucrose in TBS containing 1.5–2 μg isotopically labeled fibronectin, 6 ml of 30% sucrose in TBS, and 4 ml of TBS. After centrifugation for 16 h at 200,000 *g* at 20°C, the contents of the tubes were removed by an Isco fractionator and collected in 370 μl fractions. Aliquots of 50 μl from each fraction were used for radioactivity measurements by scintillation counting.

RESULTS

HTC cells, which have been used in previous studies (27, 28), were found to have a rather weak interaction with the substratum and between one another when grown in monolayer culture. The cells remain isolated and loosely attached to the culture dish and would not form confluent monolayers. When we established new cloned cell lines from the wild-type SR-HTC cells (27), we observed several clones that exhibited a preference to grow in clusters. One of these cell lines was selected and used in this study. The cells responded to dexamethasone with an increase in adhesiveness to the substratum, as has already been reported for the original SR-HTC cells (12), and also with tighter cell–cell interaction. This feature is most apparent in the altered morphology of the monolayer (Fig. 1). This enhanced adhesion and spreading of HTC cells after dexamethasone treatment is reminiscent of the morphological alterations seen in primary hepatocytes when fibronectin is provided as culture substratum (5). Possible explanations for these dexamethasone-induced phenomena include: (*a*) an increased amount of fibronectin in the HTC cell culture, (*b*) an improved ability of the cells to utilize the fibronectin present in the culture medium, or (*c*) an expression of membrane properties that are independent of fibronectin.

To differentiate between these possibilities, we first analyzed the effect of the steroid hormone on the pattern of proteins secreted by the hepatoma cells into the culture medium. As shown in Fig. 2, within 24 h dexamethasone elicited a drastic change in the qualitative and quantitative composition of fucose-labeled medium proteins. Some of these alterations were identical to those detected in other cell lines of SR-HTC cells (27). Besides the increased synthesis and secretion of the two glycoproteins, gp50 and α_1-acid glycoprotein (= gp35–50) (28), a third glycoprotein species with apparent molecular weight of 230,000 was also present in an elevated amount (marked with *FN* in Fig. 2). To demonstrate that this spot represents a protein with properties of fibronectin, we collected the culture media from HTC cells metabolically-labeled with [^{3}H]leucine and chromatographed it on a gelatin–Sepharose column. A steroid-dependent increase in the radioactivity recovered in the gelatin-

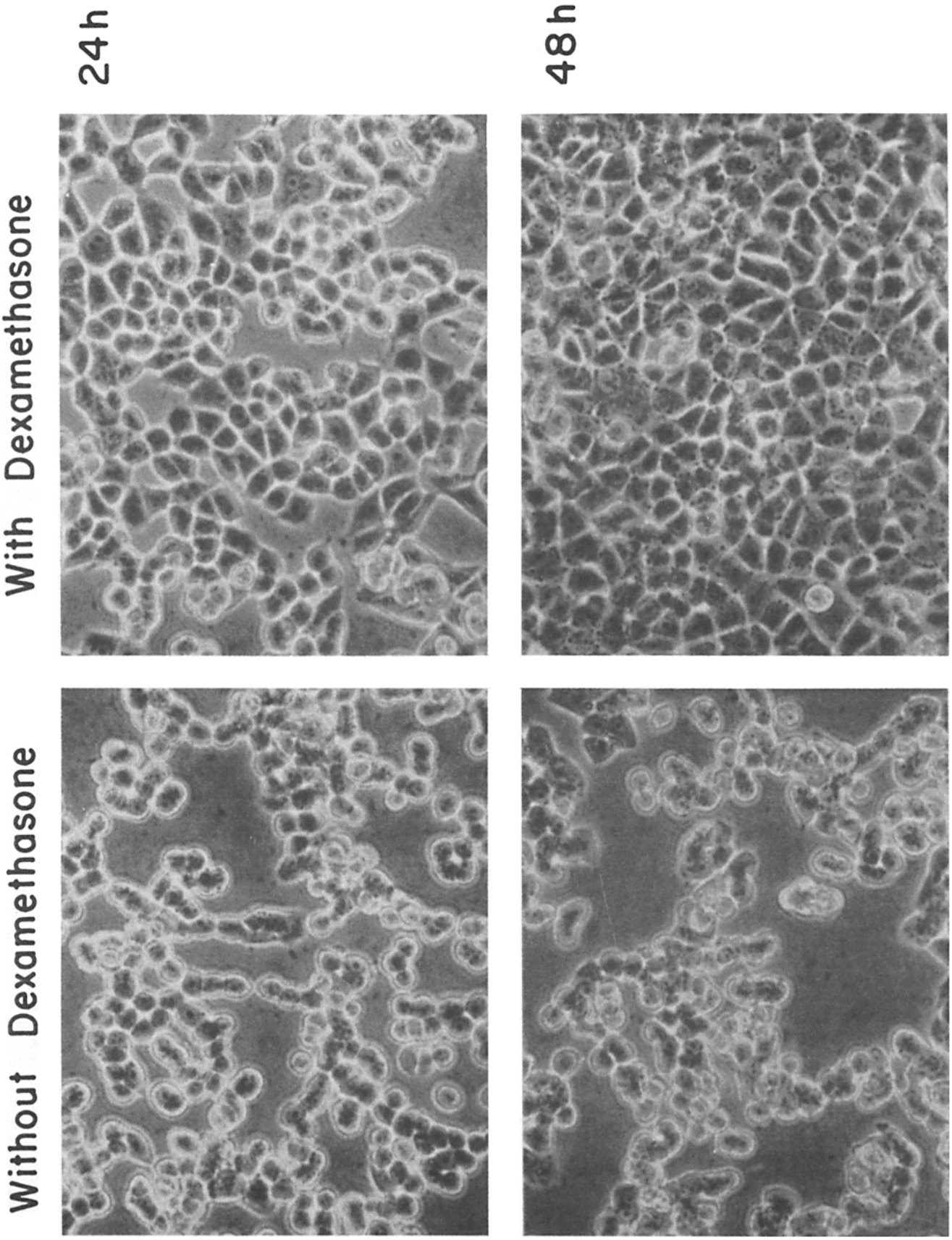

FIGURE 1 Effect of dexamethasone on the morphology of HTC cell monolayers. HTC cells were grown for 48 h in monolayer culture. Fresh culture media were then added. To one culture 1 μM dexamethasone was added. After *24* and *48 h*, the cells were photographed under a phase-contrast microscope, × 250.

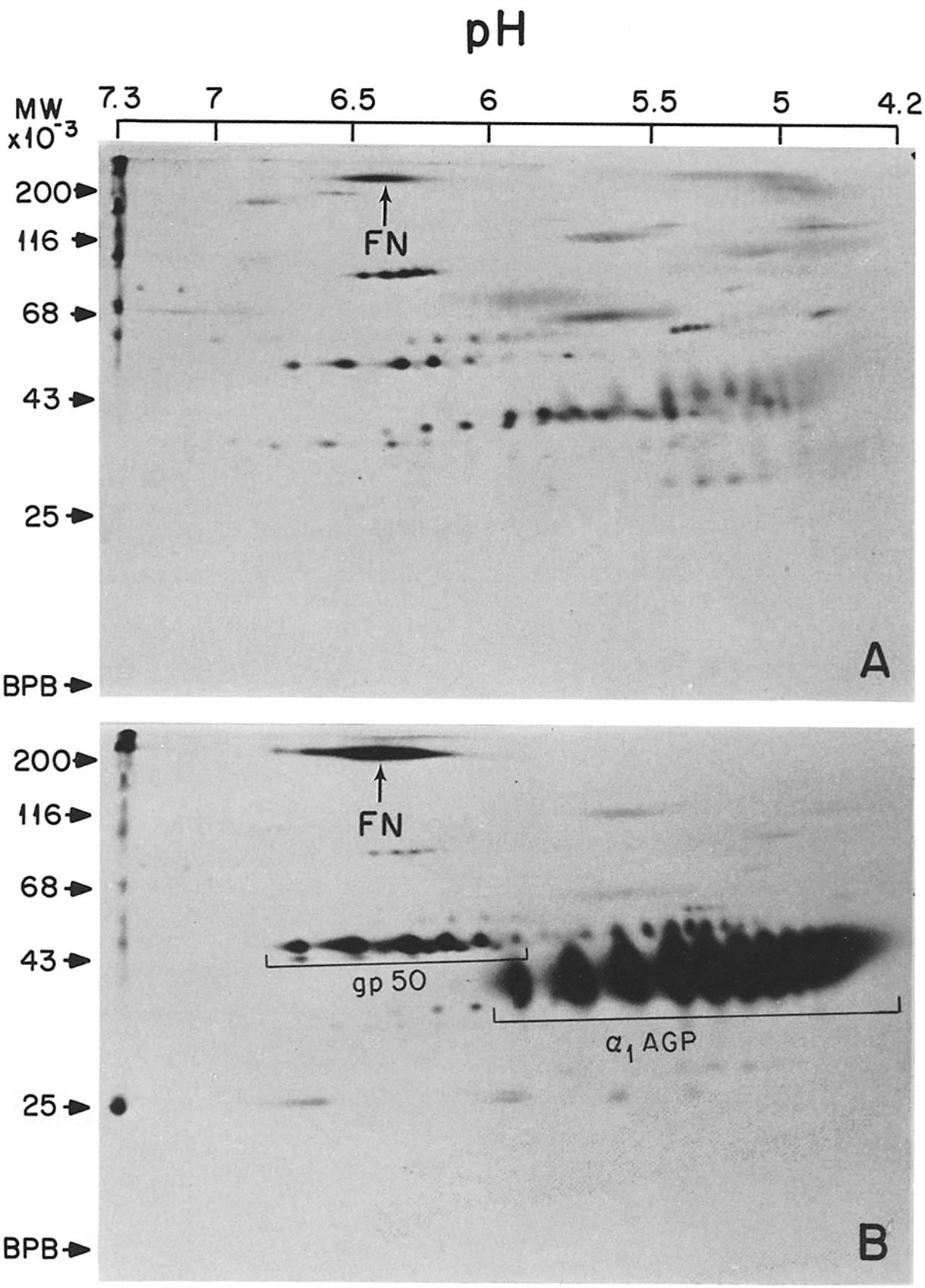

FIGURE 2 Glycoproteins synthesized and secreted by HTC cells. Confluent monolayers of HTC cells, (*A*), control and (*B*), pretreated for 24 h with dexamethasone, were metabolically labeled for 24 h with in serum-free medium containing [^{3}H]fucose (100 μCi/ml). Aliquots of 50 μl of the cell-free culture medium, containing 250,000 and 490,000 acid-insoluble cpm, respectively, were subjected without further treatment to two-dimensional separation on polyacrylamide gels. The fluorographs are shown after a 3-wk exposure. *FN*, fibronectin; *gp*, glycoprotein; α, *AGP*, α_1-acid glycoprotein. *MW*, mol wt; *BPB*, bromphenol blue.

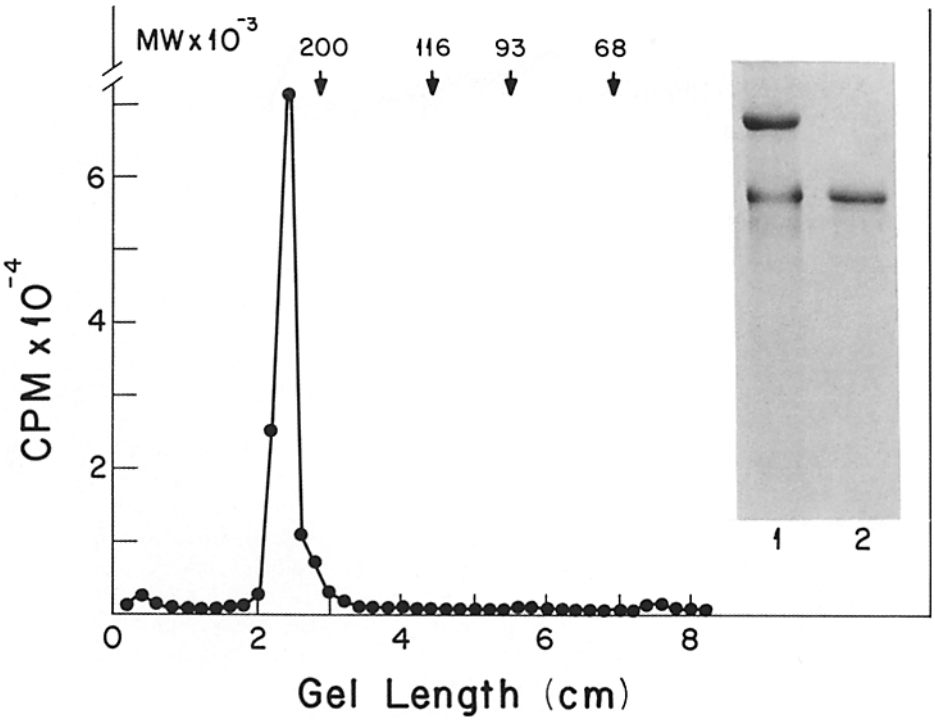

FIGURE 3 Electrophoresis of HTC-cell fibronectin after chromatography on gelatin-Sepharose. HTC cells, treated with dexamethasone, were labeled with [^{3}H]leucine (see Table I). The media were chromatographed on 1-ml columns of gelatin-Sepharose. The gelatin-bound material was eluted and one-half was separated on a 7.5% polyacrylamide gel. The distribution of the radioactivity on the gel was determined in 2-mm thick slices. The inset shows the fluorographic picture of the same fibronectin preparation separated on a 5% polyacrylamide gel either under nonreducing (*1*) or reducing (*2*) conditions. *MW*, mol wt.

bound fraction was observed. PAGE of the affinity-purified material under reducing conditions revealed that the majority of the radioactivity appeared in a single band migrating with M_r 230,000, and under nonreducing conditions in two bands, a minor one at M_r 230,000 and a major one at M_r 450,000 (Fig. 3). The same molecular weights are found for the fibronectin purified from rat plasma (data not shown). These values are in agreement with those molecular weights reported for the monomeric and dimeric forms of plasma fibronectin from other sources (17, 29). Quantitative determination of the radioisotope in the gelatin-bound material (Table I) reveals that dexamethasone treatment elicits a two- to fourfold increase in the relative synthesis and secretion of that protein, depending upon the type of precursor provided.

The use of radioactive metabolites to determine the influence of the glucocorticoid on the fibronectin synthesis is, however, complicated by the effect of the hormone on the uptake of the precursors (see legend to Table I and references 10, 27). Although valid estimates of the hormone effect on fibronectin production can be obtained by expressing the measurements as relative values, we determined the absolute amount of fibronectin secreted by HTC cells using immunological means. To do so, we immunized a rabbit with the secretory proteins of dexamethasone-treated HTC cells. The antiserum obtained was used to analyze the antigenic composition of conditioned HTC-cell culture medium by crossed immunoelectrophoresis (Fig. 4). The antigens in the three major precipitin lines were identified by reelectrophoresis of the material recovered from crossed immunoelectrophoresis plates on two-dimensional polyacrylamide gels (data not shown). The migration of affinity purified rat-plasma fibronectin on crossed immunoelectrophoresis plates is identical to that of HTC cells (Fig. 4*C*). Both fibronectins share all antigenic determinants as judged from tandem crossed-immunoelectrophoresis analysis and they yield the same peak area per μg of applied protein (data not shown). Furthermore, we could not detect any cross reaction of the antiserum with bovine or human plasma fibronectin.

TABLE I

Effect of Dexamethasone on the Synthesis and Secretion of Fibronectin in HTC Cells

Experiment	Analyzed fraction: Cell proteins	Medium proteins	Fibronectin
Metabolic labeling* with:			
[^{3}H]Leucine‡			
Control	52.4	2.3	0.17
	60.0	1.9	0.09
Dexamethasone	35.2	2.7	0.71
	37.0	3.1	0.84
[^{3}H]Fucose			
Control	3,790	700	17
	3,890	580	12
Dexamethasone	3,510	1,210	59
	3,670	1,050	50
Immunoelectrophoresis§			
Control			7.7 ± 1.9
Dexamethasone			20.4 ± 2.6

* Confluent monolayers (10 cm^2) were treated with or without dexamethasone for 24 h. The cells were then labeled for 24 h in serum-free medium containing either [^{3}H]leucine (100 μCi/ml) or [^{3}H]fucose (100 μCi/ml). The fibronectin present in the culture fluid was purified by chromatography on gelatin-Sepharose followed by electrophoresis on 7.5% polyacrylamide gel under reducing conditions (see Fig. 3). Values from two identically treated monolayers are shown and represent specific radioactivities (cpm/μg of total cell proteins).

‡ Treatment of HTC cells with dexamethasone for 24 h causes a reduction in the uptake of leucine. When the uptake of [^{3}H]leucine within 30 min was measured, using the conditions described above and in Materials and Methods, control cells have a value of 810 cpm/μg and dexamethasone treated cells a value of 690 cpm/μg.

§ Confluent monolayers (75 cm^2) were treated with or without dexamethasone for 24 h. The cells were then incubated for an additional 24 h in fibronectin-free medium. The media were concentrated 10-fold and analyzed by crossed immunoelectrophoresis as described in Materials and Methods. The amount of fibronectin was determined and expressed in μg fibronectin/24 h/mg of total cell-protein. The values represent the mean and standard deviation of four independently conducted experiments.

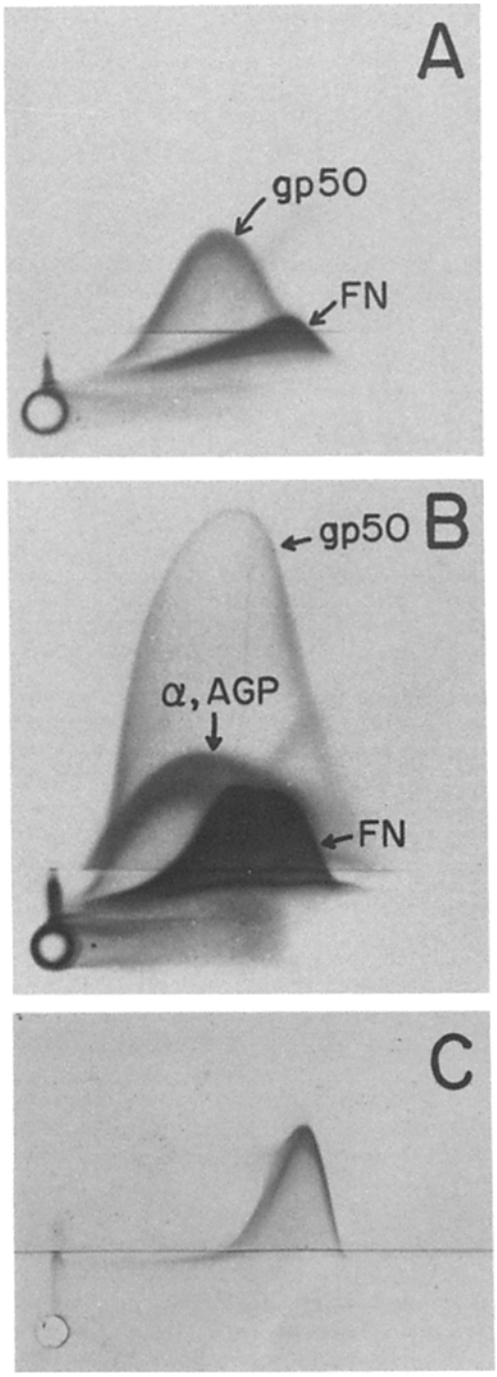

FIGURE 4 Crossed immunoelectrophoretic analysis of the secreted glycoproteins of HTC cells. HTC cells, (*A*), control, and (*B*), pretreated for 24 h with dexamethasone, were metabolically labeled for 24 h with fibronectin-free medium containing [^{3}H]fucose (10 μCi/ml). The media were concentrated 10-fold and aliquots of 15 μl separated by crossed immunoelectrophoresis. The radioactive patterns were visualized by fluorography (1-wk exposure). In *C*, 0.5 μg of affinity purified rat-plasma fibronectin was analyzed. The gel plate was stained with Coomassie Blue. *gp* 50, glycoprotein with M_r 50,000.

The immunoelectrophoretic analysis and quantification of fibronectin in several HTC-cell medium preparations indicate a threefold increase in the fibronectin concentration after treatment of HTC cells with dexamethasone (Table I). This finding agrees closely with the results of the metabolic labeling studies.

Although HTC cells release more fibronectin into the surrounding milieu after exposure to dexamethasone, monolayers of control or hormone-treated cells appear to bind little or no fibronectin, as judged by the very low level of detectable fluorescence when the cells were probed *in situ* with fluorescein-labeled antifibronectin immunoglobulins (Fig. 5 *A* and *B*). Nor does the addition of rat-plasma fibronectin to dexamethasone-treated HTC cells lead to an enhanced fluorescence (Fig. 5 *C*). The absence of externally bound fibronectin was confirmed by cell-surface labeling procedures. The treatment of HTC-cell monolayers with either lactoperoxidase-Na ^{125}I or neuraminidase-galactose oxidase-NaB[^{3}H]$_4$ did not result in labeling of a protein comigrating with fibronectin on two-dimensional polyacrylamide gels (data not shown, see similar results in references 30, 31).

To determine whether SR-HTC cells indeed do not bind fibronectin and/or whether the fibronectin produced by HTC cells lacks actual cell-binding activity, we purified fibronectin known to have all binding activities (see reference 17) and that could also be metabolically labeled in a manner comparable to that of HTC cells. We chose WI-38 human-diploid fibroblasts as a source of medium fibronectin, because among all fibroblast-cell lines these cells were found to synthesize the greatest amount of cell-bound and secreted fibronectin (32). Using [^{3}H]-fucose for metabolic labeling and gelatin affinity chromatography, we obtained fibroblast fibronectin, which appeared on two-dimensional gel as a single spot and did not differ significantly in its electrophoretic mobility from the hepatoma product (data not shown). These two fibronectin preparations were subsequently used in bioassays (33, 34) to test the binding activites of both the cells and the fibronectins (Table II). To exclude the possibility that there are species-specific binding properties, we also studied ^{125}I-labeled rat-plasma fibronectin (Experiment II in Table II). It appears that HTC cells, with or without dexamethasone treatment, do not bind appreciable amounts of either their own fibronectin or that of fibroblasts. In addition, the HTC-cell-derived fibronectin was bound to WI-38 cells much less efficiently than the fibroblast or rat-plasma fibronectin.

Despite the lack of binding of soluble fibronectin of HTC cells to cells, the possibility still exists that this protein can promote cell attachment and spreading. Therefore, we coated culture substrata with fibronectin from either rat plasma or HTC cells. Using WI-38 fibroblasts and dexamethasone-treated HTC cells as test cells for the activity of the prepared surfaces, we observed that the adhesion of fibroblasts was promoted only by plasma-derived fibronectin and that this activity could be abolished by pretreatment of the coated plate with antibodies against fibronectin (Fig. 6). The attachment and spreading of HTC cells, however, were not significantly influenced by the nature of the culture substratum. A similar test employing hormone-untreated HTC-cells yielded the same results (data not shown).

To corroborate the partial activity of HTC-cell fibronectin using another system, we took advantage of the observation of Kleinman and co-workers (35, 36) that fibronectin has a specific affinity to more highly sialylated gangliosides. This hypothesis is attractive with respect to the hepatoma-cell system, for HTC cells do not contain any ganglioside species with more than one sialic acid residue per molecule (26). The lack of these membrane constituents might explain the absence of fibronectin binding, despite the observation that highly sialylated gangliosides were minimally present in nontransformed fibroblasts,

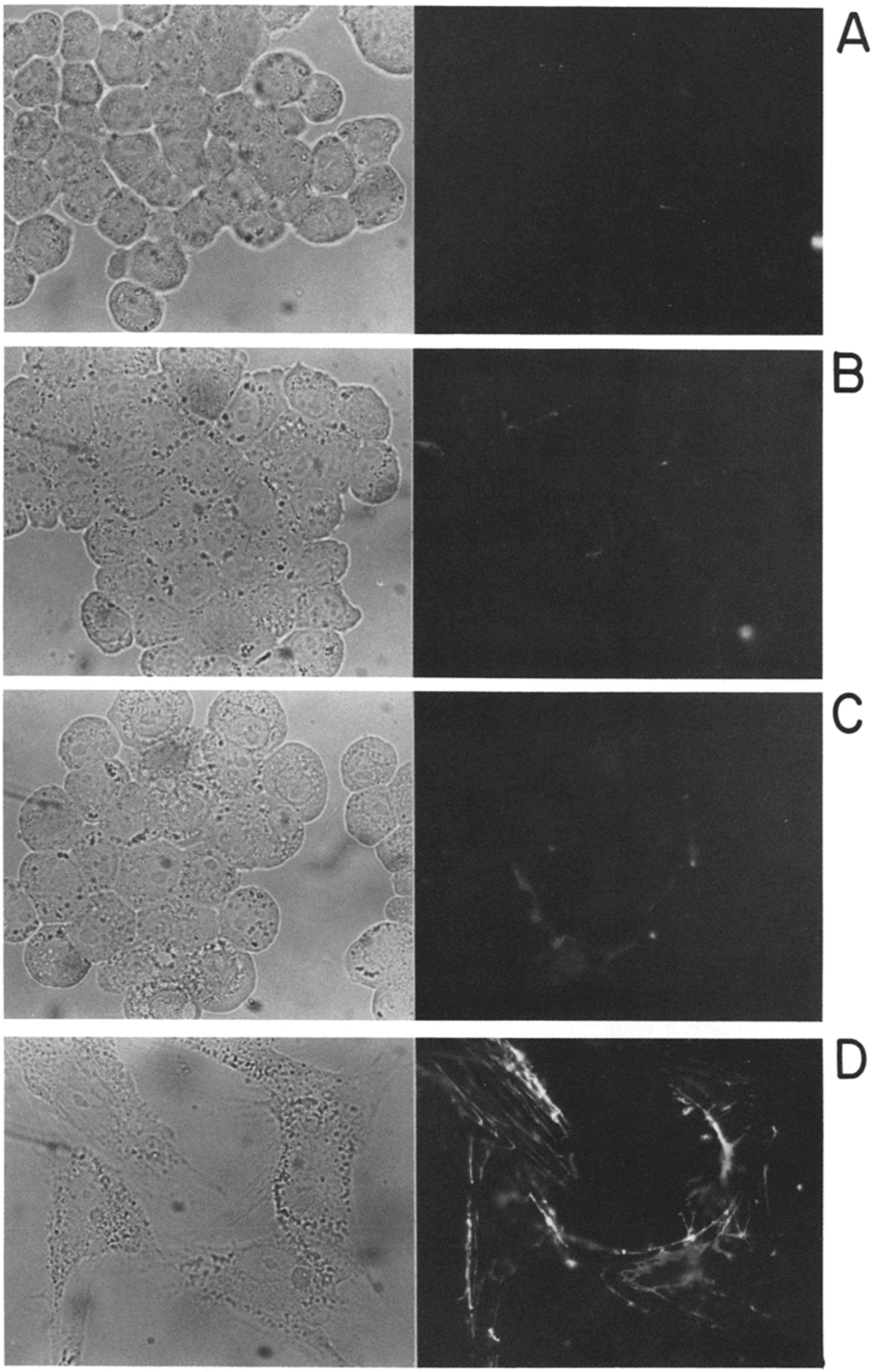

FIGURE 5 Cell-surface localization of fibronectin by fluorescent immunoglobulins. HTC cells, control (*A*) and treated for 48 h with dexamethasone (*B* and *C*) and fetal-rat fibroblasts (*D*), were cultured on glass cover slips. To the medium (2 ml) of the cells in *C*, 100 μg of affinity purified rat-plasma fibronectin was added after 24-h hormone treatment. The presence of surface-bound fibronectin was assessed by fluorescent antibodies against rat fibronectin. *Left*, light-microscopic picture, × 400; and *right*, the fluorescent image of the same cells.

TABLE II

Association of Exogenously Added Fibronectin to HTC Cells and WI-38 Fibroblasts

Cells tested	Radioactive fibronectin bound: HTC-cell fibronectin	Radioactive fibronectin bound: WI-38-cell fibronectin	Radioactive fibronectin bound: Rat-plasma fibronectin
	μg/mg of total cell protein		
Experiment I			
HTC cells			
Control	0.7	0.5	
	0.9	0.5	
Dexam ısone-treated	0.5	0.5	
	0.6	0.9	
WI-38 Fibroblasts	2.8	9.1	
	3.3	13.5	
Experiment II			
HTC cells			
Control	0.1		0.1
	0.1		0.1
Dexamethasone-treated	0.1		0.1
	0.2		0.1
WI-38			
Control	0.7		8.7
	0.7		8.4
With excess rat-plasma fibronectin	0.5		0.5
	0.7		0.6

To obtain metabolically labeled fibronectin for binding studies four flasks (150 cm^2), each of confluent HTC cells treated with dexamethasone, and WI-38 fibroblasts were labeled with [^{3}H]fucose (10 μCi/ml) for 48 h in fibronectin-free medium. The synthesized and secreted fibronectins were purified by gelatin affinity chromatography. To test the binding specificity (*Experiment II*), we prepared ^{125}I-labeled fibronectin from rat plasma. The final preparations had the following specific radioactivities: HTC-cell fibronectin, 20,700 cpm/μg (Experiment I); and 8,400 cpm/μg (Experiment II); WI-38 fibronectin: 4,300 cpm/μg; and rat plasma fibronectin: 87,000 cpm/μg. HTC cells, with or without prior dexamethasone treatment, as well as WI-38 fibroblasts were released from monolayers by trypsin. The cells were washed once with fibronectin-free medium and resuspended in the same medium yielding densities of 1×10^6 HTC cells/ml and 5×10^5 WI-38 fibroblasts/ml. 2 ml of the cell suspensions were placed into 25-cm^2 plastic dishes. Before use, these culture dishes were incubated with 5 ml fibronectin-free medium for 2 h at room temperature. To each culture (in duplicates), 15 μg of radiolabeled fibronectin was added. We tested the binding specificity of fibronectin to fibroblasts by adding in Experiment II, to some of the WI-38 cultures, ^{125}I-labeled rat-plasma fibronectin together with an excess (250 μg) of unlabeled fibronectin. After 24 h in culture, the cells attached to the dishes were washed three times with PBS. The acid-precipitable radioactivities bound to the cell monolayers as well as the total amount of cell protein were determined. Based on the known specific activities of the fibronectin preparations, the μg amounts of fibronectin bound per mg of total cell protein were calculated. To estimate the degradation of radioactive fibronectin, the cells from the culture media after a 24-h incubation were passed through gelatin-Sepharose columns and the bound material analyzed on polyacrylamide gels. In all cases, 92–96% of the radioactivities present in the media after 24 h were still associated with intact fibronectin.

and with the subsequent conclusion by Rauvala et al. (37) that these plasma-membrane constituents do not act as true cell-surface receptors for fibronectin. Nevertheless, we used this peculiar binding property to analyze the activity of hepatoma and fibroblast fibronectin. The assay system consisted of phosphovesicles carrying higher sialylated gangliosides that were centrifuged through a layer of [^{3}H]fucose-labeled fibronectin. If binding of fibronectin to the vesicles takes place, it can be detected by comigration of tritium with the phospholipid vesicles (Fig. 7). To mark precisely the position of the isopycnically concentrated vesicles in the centrifuge tube, we utilized radiolabeled vesicles prepared by addition of trace amounts of ^{32}P-labeled phospholipids to the reconstitution mixture. Although this assay system allows only a qualitative assessment of binding activity, it clearly shows that the fibronectin from HTC cells, unlike that from fibroblasts, lacks any detectable interaction with the artificial membrane. This result, combined with the previous data in Table II, indicates that although it is structurally similar to fibroblast fibronectin, hepatoma fibronectin shares only partial activity with that for fibroblasts.

To study further the structural properties of HTC-cell fibronectin, we needed for appropriate comparison a fibronectin molecule that is synthesized by nontransformed cells (34). Because the HTC cells are of hepatic origin, the logical source of such fibronectin would be parenchymal cells of the liver. Previous labeling experiments have shown, however, that primary cultures of fully differentiated adult rat hepatocytes, whether or not they are treated with dexamethasone, do not synthesize or secrete detectable amounts of fibronectin (28). Therefore, we analyzed primary cultures of fetal rat hepatocytes. As shown in Fig. 8*A*, fibronectin represents a significant portion of the proteins synthesized and secreted by these cells. We have confirmed that this protein binds to gelatin and is recognized by the anti-rat fibronectin immunoglobulins (data not shown). For structural comparison, [^{35}S]methionine-labeled, monomeric fibronectin from fetal rat hepatocytes and from HTC cells were recovered from two-dimensional polyacrylamide gels and analyzed by partial proteolytic mapping (Fig. 8*B*). To demonstrate the validity of proteolytic comparison for proteins that are shared between fetal hepatocytes and the HTC cells, we included a similar digestion of gp50, a glycoprotein that is, like fibronectin, regulated by dexamethasone in hepatoma cells and is exclusively expressed by fetal hepatocytes (for data on adult hepatocytes, see reference 28). The peptide patterns of the proteins from the two former sources show almost perfect homology indicating structural similarity of the polypeptide moieties. Close inspection of the fragmentation pattern of the fibronectin proteins, however, reveals one band differing in mobility (indicated by arrow in Fig. 8*B*). Whether this structural difference is connected with the reduced biological activity of the hepatoma fibronectin remains to be analyzed. Furthermore, we do not yet have sufficient data to determine whether or not fibronectin synthesis and secretion in fetal hepatocytes are regulated by glucocorticoids.

DISCUSSION

We have attempted to determine whether fibronectin plays any role in the adhesion and cell–cell interaction of rat hepatoma cells. Two distantly related phenomena have stimulated this line of investigation: first, nontransformed adult rat hepatocytes require for adhesion and spreading either collagen (3) or fibronectin (5), both components of the liver matrix (2), and secondly, hepatoma cells exhibit a marked increase in adhesion to the substratum upon dexamethasone treatment (12). An additional intriguing observation is that dexamethasone elicits in HTC cells an increased synthesis and secretion of a glycoprotein with gelatin-binding activity, similar in size and charge to the soluble fibronectin of fibroblasts and containing the same antigenic determinants as rat plasma fibronectin. Two results strongly suggest that the steroid-induced adhesion and spreading properties of HTC cells are not mediated by fibronectin. First, in HTC cells, no significant amount of fibronectin protein is accessible to immunoglobulin probing (Fig. 4) or to radioactive modification by cell-surface labeling techniques.

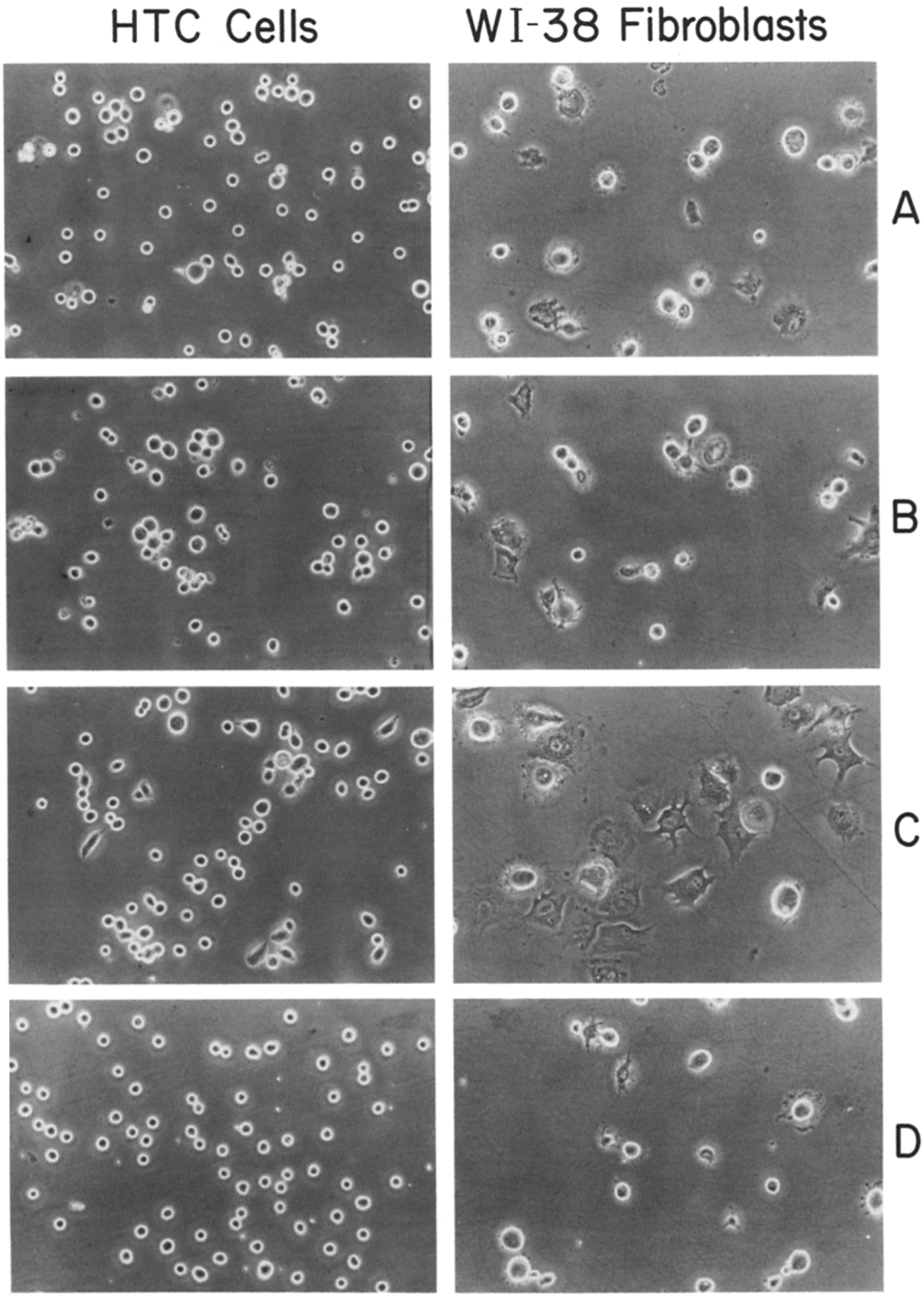

FIGURE 6 Attachment of cells to fibronectin-coated culture surfaces. HTC cells, treated for 48 h with dexamethasone, and WI-38 fibroblasts were incubated in nontreated plates (*A*) and plates coated with HTC cell fibronectin (*B*), with rat-plasma fibronectin (*C*), and with rat-plasma fibronectin exposed to antifibronectin immunoglobulin (*D*). × 240.

HTC CELL-FN
FIBROBLAST-FN
WITH GANGLIOSIDES
WITHOUT GANGLIOSIDES
^{3}H-RADIOACTIVITY (cpm x 10^{-2})
^{32}P-RADIOACTIVITY (cpm x 10^{-3})
FRACTION
1
2

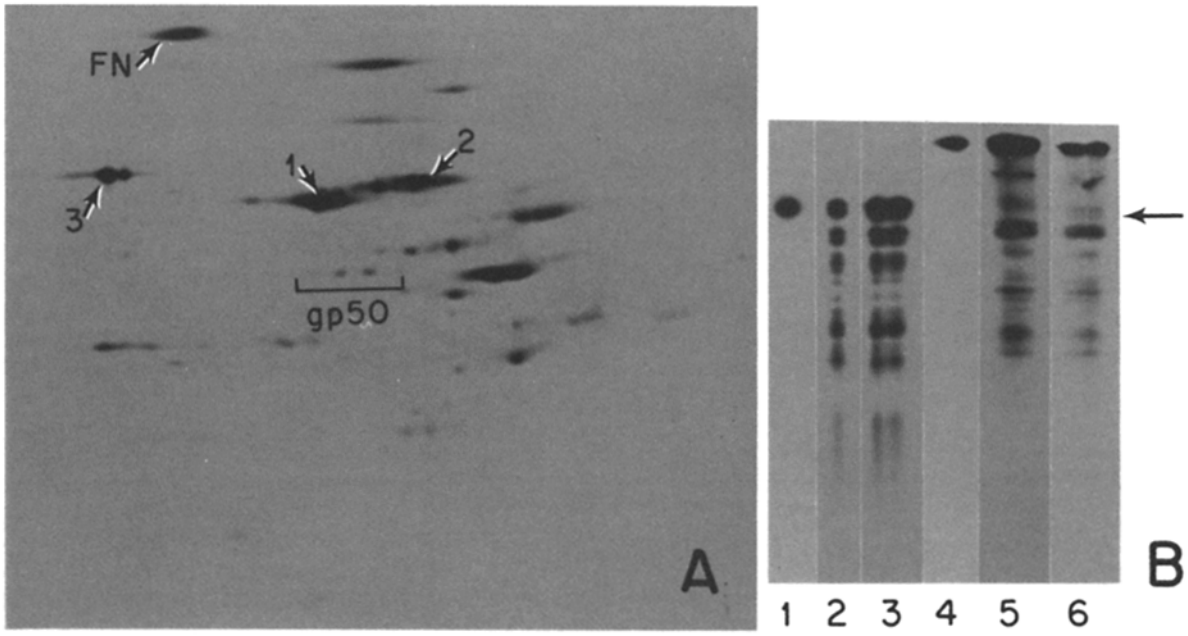

FIGURE 8 Analysis of the proteins secreted by primary fetal hepatocytes. *A*: After 4 d in culture, primary fetal rat hepatocytes in a 3.5-cm dish were labeled for 24 h with [^{35}S]methionine in serum-free medium. An aliquot of the culture medium, containing 200,000 acid-insoluble cpm, was separated by two-dimensional polyacrylamide gels. The pattern was visualized by fluorography (2-wk exposure). Arrows indicate the position of: albumin (*1*), α_1-fetoprotein (*2*), transferrin (*3*), glycoprotein with M_r 50,000 (*gp50*) and fibronectin (*FN*). *B*: Concentrated media containing [^{35}S]methionine-labeled proteins of fetal rat hepatocytes and dexamethasone-treated HTC cells (each preparation containing 1.2 × 10^6 cpm) were separated as in *A* and the spots corresponding to fibronectin and to *gp50* were analyzed by partial proteolytical digestion with *S. aureus* protease on a 15% polyacrylamide gel (19). The lanes represent *1*, rat-hepatocyte *gp50* without protease; *2*, rat-hepatocyte *gp50*; *3*, HTC-cell *gp50* with 3 N-glycans (see reference 28); *4*, rat-hepatocyte fibronectin without protease; *5*, rat-hepatocyte fibronectin; *6*, HTC-cell fibronectin. To achieve comparable intensities, the various lanes were exposed for periods ranging from 2 to 14 d. Arrow indicates the difference in the peptide pattern of HTC-cell fibronectin.

However, using the same labeling techniques for fibroblast monolayers, fibronectin appears as the most prominently labeled component (38, 39). Second, neither exogenously supplied fibroblast fibronectin nor its own fibronectin were bound by HTC cells (Fig. 5 and Table II). The absence of fibronectin on the cell surface of HTC cells is not unique to this cell type, as two other hepatoma-cell lines, mouse Hepa II cells and rat H-35 cells, do not display surface-bound fibronectin (H. Baumann, unpublished observations). Furthermore, unlike HTC cells, these two hepatoma-cell lines do not synthesize or secrete detectable amounts of fibronectinlike proteins under any known hormonal culture conditions. From these data, the specific identity of the cell-surface component(s) responsible for adhesion either in HTC cells or other hepatoma cells is not apparent. Moreover, we have at the present time no evidence whether these hepatoma cell lines synthesize and secrete a protein that promotes cell attachment, but is structurally different from fibronectin and does not bind to gelatin (40).

Because a role for fibronectin in the adhesion of nontransformed hepatocytes has not been clearly established (3–5), speculation about a possible loss of fibronectin recognition by hepatoma cells due to transformation is not warranted. However, that transformation can indeed lead to reduced fibronectin-binding has been demonstrated for hamster fibroblasts (34).

Although fibronectin synthesized by HTC cells binds appropriately to gelatin, it appears not to be a completely functional fibronectin, because it binds only partially to fibroblasts and not at all to ganglioside-containing phospholipid vesicles. Whether the binding activities for other molecules such as glycosaminoglycans (41), fibrin (42), DNA (43), *S. aureus* (44), or actin (45) are still preserved, remains to be determined. The occurrence of functionally defective fibronectin is not unique to HTC cells, as there are reports of the production of similarly inactive fibronectin by senescent human fibroblasts (46) and of the presence of defective plasma fibronectin in a patient with Ehlers-Danlos syndrome (47). This report of a fibronectin protein with partial activity provides the opportunity to study the specific structural or chemical requirements for binding activity.

During the process of transformation and culture selection, HTC cells have retained few properties that are considered to be liver-specific; most noted are the glucocorticoid-regulated

FIGURE 7 Binding of fibronectin to ganglioside-containing phospholipid vesicles. Phospholipid vesicles, with or without gangliosides, were prepared as described in Materials and Methods. The vesicles that were used in the separations shown in the top panels, contained in addition 38,000 cpm of [^{32}P]phospholipids. Aliquots of the vesicle preparations were placed into ultracentrifuge tubes overlaid with 0.5 ml of sucrose solution containing either 1.6 μg of HTC-cell fibronectin (=35,000 cpm) or 1.8 μg of fibroblast fibronectin (=7,300 cpm) and centrifuged as outlined in Materials and Methods. After isopycnic centrifugation and fractionation of the gradients, the radioactivities present in 50-μl aliquots of each fraction were determined. *Inset*, left panel shows the thin-layer pattern of the gangliosides incorporated into the phospholipid vesicles (*1*) next to the GM_1 ganglioside standard (*2*).

activity of tyrosine aminotransferase (9) and synthesis of α_1-acid glycoprotein (28). Fibronectin, however, is barely detectable as a secretory product of primary adult hepatocytes from either normal or dexamethasone-treated rats (27, 28). The low levels occasionally observed in the culture fluid of primary hepatocytes (48) can be attributed to contaminating fibroblasts in the hepatocyte preparation. For this reason, HTC cells are an interesting system, not only because of the relatively large quantities of soluble fibronectin produced, but also because of the glucocorticoid-regulated synthesis. There is a report that dexamethasone treatment of hepatocytes derived from newborn rats leads to an increased amount of cell-surface localized fibronectin as judged by fluorescent immunological staining (49). Similarly, a glucocorticoid-dependent enhancement of immunoreactive fibronectin on the surface of transformed human fibroblasts has been observed (50). In neither case, however, has it been determined whether the glucocorticoid treatment affected the synthesis of fibronectin or the affinity of the cell to bind fibronectin. The occurrence of dexamethasone-regulated synthesis of fibronectin in HTC cells, therefore, could be either a property of developmentally deregulated hepatocytes or the consequence of transformation. Future experiments with primary fetal hepatocytes, which are found to synthesize fibronectin, and long-term tissue cultures of primary adult hepatocytes, will give a definite answer. The reason for the partial loss of activity of HTC-cell fibronectin cannot at the present stage be elucidated. One remaining possibility, as yet untested, is offered by the observation that secondary modification of newly synthesized glycoproteins in HTC cells is different from that in adult hepatocytes (28). However, the findings that carbohydrate-free fibronectin (51) or fibronectin with altered oligosaccharide structure (34) exhibit normal activities, make this explanation appear less likely.

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