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Human Fibrosarcoma Cells Produce Fibronectin-Releasing Peptides

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A sensitive radioimmunoassay, specific for human fibronectin, was used to measure the ability of certain biologically active polypeptides to release fibronectin from cultured human lung fibroblasts into their culture media.

Concentrated, serum-free culture supernatant from a human fibrosarcoma cell line was fractionated by gel filtration chromatography in the presence of acetic acid. Various polypeptides with molecular weights between 46,000 and 6,000 were tested for their ability to release fibronectin from cells. The column fraction, containing polypeptides with an apparent molecular weight of 10,000, exhibited the ability to rapidly release fibronectin from target cells. The activity could be inhibited by phenylmethyl sulphonylfluoride. Several other hormonal factors, tested in parallel with the column fractions, failed to show this effect. The 10,000 dalton molecular weight polypeptides may represent a family of cellular gene products responsible for maintenance of low levels of surface associated fibronectin in fibrosarcoma cells and thus be related to their infiltrating properties by preventing the formation of the extracellular matrix.

Key words: **fibrosarcoma culture media, gel permeation chromatography, fibronectin-radioimmunoassay, fibronectin-releasing peptides**

Transformed cells frequently show a characteristic loss of cell surface-associated fibronectin [1–3]. Certain biochemically known agents can induce the release of fibronectin from cells. Several proteases release fibronectin from the cell surfaces [4–6], also human thrombin from human fibroblasts [7]. On the other hand, fibronectin is released from cells in apparently intact form by either urea [8] or cytochalasin B, a microfilament disrupting agent [9, 10]. Certain tumor promoters, namely, the biologically active phorbol esters, are also capable of inducing a rapid release of fibronectin from cells [11] but thus far no cell-derived factors have been identified.

Abbrevations: EGF, epidermal growth factor; SGFs, sarcoma growth factors; MSA, multiplication stimulating activity; SDS, sodium dodecyl sulphate; K, 10^3 (in molecular weights); BSA, bovine serum albumin; PBS, phosphate buffered saline; PMSF, phenylmethyl sulphonyfluoride; FCS, fetal calf serum.

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Transformed cells and tumor cell lines produce factors assumed to be important for their altered growth properties. The production of certain growth factors may explain the molecular background and mechanisms needed for the invasive properties of different types of tumors [12]. Established lines of human fibrosarcomas have been used to study the altered properties of malignant cells. An established human fibrosarcoma cell line (8387) [13] has been shown to produce a family of polypeptides with multiplication stimulating activity (MSA) [14]. This fibrosarcoma line grows in soft agar, is tumorigenic in nude mice [13] and has low levels of surface-associated fibronectin [our unpublished observation]. Fibrosarcoma cells also produce increased amounts of plasminogen activators [15, 16], and express reduced amounts of surface-associated fibronectin [1, 2]. Very little, however, is known about the mechanisms of fibronectin release from transformed cells. The tissue invasiveness of malginant cells may, at least to some extent, be a result of their ability to pervade fibronectin-containing structures that are probably important in the formation of the extracellular matrix. In this report we describe how fibroblast-associated fibronectin can be released from the cells by a partially purified, 10,000 molecular weight polypeptide(s) that is apparently of proteolytic character, obtained from the serum-free culture media of the established human fibrosarcoma line (8387).

MATERIALS AND METHODS

Isolation of Peptides From Human Fibrosarcoma Culture Media

Supernatant fluids from a human fibrosarcoma cell line (8387) were collected using serum-free Waymouth's medium. The low molecular weight peptides were partially purified from the supernatants by a method that utilizes the natural affinity of these peptides for a high molecular weight binding protein [17, 18]. The medium was ultrafiltered at neutral pH using a high flow Amicon hollow fiber apparatus (50,000 dalton exclusion) and the retentate was acidified to pH 2.3 to dissociate the peptides from their binding protein. Chromatography was carried out on a Bio-Gel P-100 column which was eluted with 1M acetic acid. Five different pooled column fractions with molecular weights varying between 46,000 and 6,000 were used for assays (see below).

Radioimmunoassay for Fibronectin

A specific antibody to human plasma fibronectin was raised by immunizing rabbits. The immunizing antigen was purified by gelatin-Sepharose affinity chromatography and DEAE-cellulose chromatography, as described previously [11, 19]. Antifibronectin antiserum produced in rabbits, was adsorbed with 2 volumes of fetal calf serum (37°C for two hours and 4°C for 18 hours) and clarified by centrifugation. This adsorption removed all cross-reacting antibodies against bovine fibronectin and medium containing 10% FCS caused no inhibition in the binding. Plasma from mouse and rat or tissue culture media from cell lines of heterologous species failed to compete in the assay. This radioimmunoassay using preadsorbed immune sera and ^{125}I-labeled human plasma fibronectin is thus specific for human fibronectin [7, 11].

Purified human fibronectin was iodinated by using 2 μg chloramine-T for 10 μg of protein in 0.4 M phosphate buffer (pH 7.6). The reaction was carried out for one minute at room temperature and then quenched with a saturated solution of tyrosine. Iodinated protein was recovered in the void volume of a Bio-Gel P-60 column. Competition radioimmunoassay was per-

formed by a double antibody precipitation method [7, 11]. In brief, the appropriate dilution of rabbit antifibronectin was chosen for the precipitation of ~50% of input radiolabeled fibronectin. The antisera was first incubated with serial dilutions of unlabeled competing test antigen samples at 37°C for 2 hours. Approximately 20,000 dpm of ^{125}I-fibronectin was then added and incubated for an additional 2 hours at 37°C. The immune complex was incubated at 37°C for 1 hour and 4°C for 3 hours with sheep anti-rabbit immunoglobulin as the second antibody. The immune precipitate was washed 3 times with cold PBS. The amount of ^{125}I-fibronectin in the precipitate was directly measured in a gamma counter.

A solution of fibronectin in 0.5% BSA served as standard. It was necessary to divide the standard and freeze in small aliquots to prevent the loss of the antigenic activity which occurs with repeated freezing and thawing [20]. The competition curves were linear between 4–40 ng of fibronectin and the variation between two identical samples tested in parallel was less than 5% [11].

Cell Cultures and the Assay for Fibronectin Release

Diploid human embryo lung fibroblasts (CCL-137) were obtained from the American Type Culture Collection, Rockville, Maryland. Cells were grown on plastic tissue culture dishes (Falcon Industries, Oxnard, California) at 37°C in Eagle's basal medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 50 μg/ml streptomycin. Cells were between passages 13 and 19. Upon reaching confluency, 3–6 days after subculture the growth medium was removed and the cells were washed thrice with the binding buffer (Dulbecco's modification of Eagle's media plus 50 mM N,N-bis-(2 hydroxyethyl)-2-aminoethanesulphonic acid and 1 mg/ml of BSA, pH 6.8). The experiments were carried out in binding buffer. After the cultures were incubated at 37°C for the time described, the medium was collected and clarified by centrifugation at 300 × g for 10 minutes and their fibronectin concentrations were determined by radioimmunoassays. The attached cells were washed 3 times with cold PBS and extracted with 500 μl of urea-Triton solution (1% Triton X-100, 6 M urea, 1 mM PMSF). The protein concentrations of the cell extracts were determined [21].

Labeling of Cells With ^{35}S-Methionine

Cultures of human lung fibroblasts were washed three times with methionine-free media and then labeled with 30 μCi/ml of ^{35}S-methionine (103 μCi/mmol, The Radiochemical Centre, Amersham, England) in methionine-free media for 4 hours. The labeling was terminated by washing the cells with media containing excess of unlabeled methionine. The cultures were then incubated in the binding buffer as shown. Upon termination of the incubation the media of the cells were collected, clarified by centrifugation (300 × g), and the polypeptides were precipitated with cold trichloracetic acid (10% final concentration) and analyzed in SDS-polyacrylamide gels.

SDS-Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide gels were prepared according to previously published methods [22, 23]. Purified plasma fibronectin (subunit molecular weight 220,000), phosphorylase b (molecular weight 94,000), bovine serum albumin (molecular weight 68,000) ovalbumin (molecular weight 45,000) carbonic anhydrase (molecular weight 29,000), myoglobin (molecular weight 17,200) and bromphenol blue were used as markers for molecular weight and mobility.

RESULTS

The Release of Fibronectin by Fibrosarcoma Derived Polypeptides

Five different pooled column fractions, isolated by gel filtration as described, were analyzed for their ability to release fibronectin from confluent cultures of human lung fibroblasts. The mobilities of the major protein bands of the pooled fractions were between 46,000 and 6,000 daltons in SDS-polyacrylamide gel electrophoresis. Equal amounts of protein were added to the serum-free media of the cells. The column fraction that contained polypeptides with an apparent molecular weight of 10,000, contained an activity that released fibronectin from the cells into their serum-free media. (Table I). Cultured fibroblasts produced and released a basal level of fibronectin into their media, and the amounts of fibronectin released from cultures treated with the other column fractions did not differ significantly from this control value.

When the 10K-peptide pool was analyzed in SDS-polyacrylamide gels, it contained a major protein band with apparent molecular weight of 10,300 and a minor band of 9,300 (Fig. 1). So far, attempts to purify these individual peptides have been unsuccessful.

To study how much of the 10K-fraction was needed to bring about the release, we used different concentrations as shown in Figure 2. Microgram quantities of the fraction were necessary suggesting either a low specific activity or that only a fraction of the total protein is active. The release of fibronectin is time dependent (Fig. 3). An increase in the fibronectin content in the media of the cells treated with 10K-peptides was seen within 30 minutes after the onset.

Analysis of the Released Molecules

To analyze the sizes of the cellular molecules released to the medium, cells were labeled with radioactive methionine and then incubated with the 10K fraction polypeptides (10 μg/ml) for 4 hours. The released polypeptides were precipitated with trichloracetic acid and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (Fig. 4). The polypeptide patterns were apparently identical. The released radiolabeled polypeptides that comigrated with the subunits of purified human plasma fibronectin and with the 220,000 molecular weight polypeptides secreted from

TABLE I. Fibronectin-Releasing Ability of Different Pooled Column Fractions*

Pool No.	Apparent mol wt.	Total fibronectin released to media (μg)	Difference between treated and control plates (μg)
1	46K–24K	1.0	0
2	14K–13K	0.8	0
3	12K	0.8	0
4	10K	5.1	4.1
5	6K	1.1	0.1
–	control	1.0	0

*Averages of duplicate dishes.

Human lung fibroblasts were cultivated on 60 mm diameter dishes and upon confluency washed 3 times with binding buffer before assay. The cells were then incubated for 4 hours in 2.5 ml of binding buffer with different pooled column fractions (5 μg/ml final concentration). The fibronectin concentrations of the media were determined by radioimmunoassays. The protein concentrations of the cultures were 422 ± 37 μg. The molecular weights given represent the mobilities of the major protein bands of the pools in SDS-polyacrylamide gels.

untreated cells, did not show signs of major proteolytic cleavages, as is seen in the case of trypsin treated cells [6], suggesting that only a minor part of fibronectin was cleaved during the treatment of the cells with 10K-peptides.

Effects of Some Hormonal Factors on the Release of Fibronectin

We analyzed the 10,000 molecular weight polypeptides produced by an established human lung carcinoma cell line (9812) as a control, as well as several known hormonal factors. None of them caused the release of fibronectin from the treated cells (Table II). The effects of some proteinase inhibitors were studied in parallel. If phenylmethyl sulphonylfluoride was present with the 10K-peptide(s), it was able to inhibit the fibronectin releasing activity of this material. Phenylmethyl sulphonylfluoride blocked the fibronectin-releasing activity of this 10K-peptide(s) at the concentration of 10 μg/ml, suggesting that the activity present in this fraction is of proteolytic character, possibly a serine protease. The activity was also inhibited by leupeptin (10 μg/ml) and soybean trypsin inhibitor (40 μg/ml).

DISCUSSION

We have shown in this study that human fibrosarcoma cells produce an activity that can release fibronectin from cultured human fibrobalsts. This protein is a small acid-stable peptide with an apparent molecular weight of 10,000 and it acts as a protease. The size of the fibronectin molecules found in the media of the cells treated with these 10K-

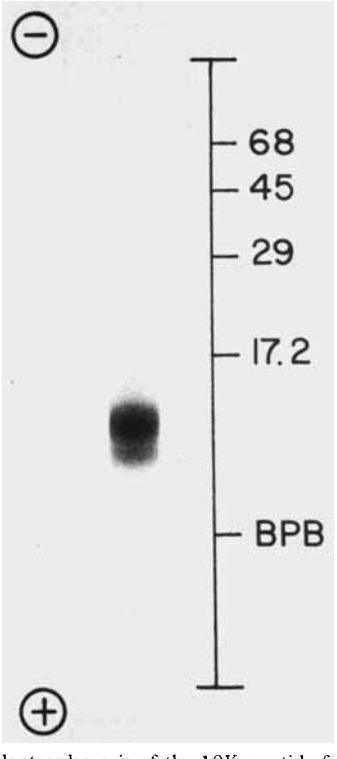

Fig. 1. SDS-polyacrylamide gel electrophoresis of the 10K-peptide fraction. A lyophilized aliquot of the column fraction was incubated in 10% SDS and 5% mercaptoethanol at +100°C for 3 minutes and analyzed in a 10% SDS-polyacrylamide gel and stained with coomassie blue. Molecular weight and mobility markers ($\times 10^3$) were bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin and bromphenol blue (BPB).

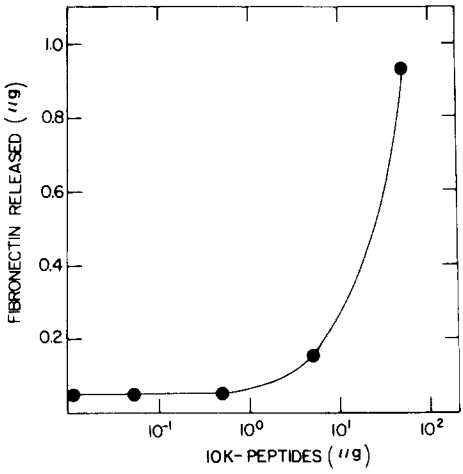

Fig. 2. The dependence of fibronectin release to increasing concentrations of the 10K-peptides (averages of duplicate dishes). Confluent cultures of human lung fibroblasts were exposed to increasing concentrations of 10K-peptides in serum-free media for 2 hours. Prior to the experiment the cultures were incubated in serum-free media for 12 hours. The supernatants were clarified by centrifugation and their fibronectin concentrations were determined by radioimmunoassays. The protein concentrations of the cultures were 206 ± 15 μg. Abscissa: the concentration of 10K-peptides (μg); Ordinate: fibronectin released into the media (μg).

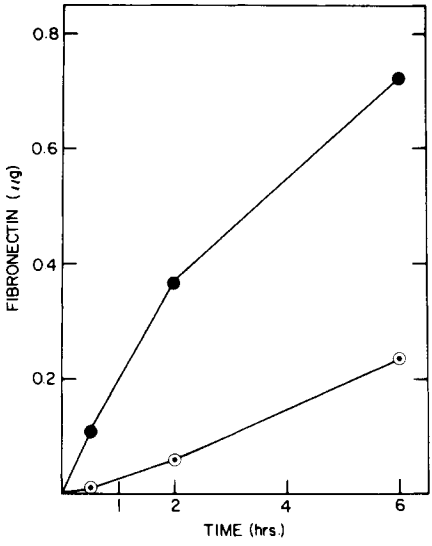

Fig. 3. Time course of fibronectin release by 10K-peptides (averages of duplicate dishes). Human lung fibroblasts were cultivated on 35 mm diameter plastic petri dishes. Confluent cultures were washed twice with binding buffer and incubated in binding buffer for 12 hours before the assay. The cultures were then washed 3 times and incubated in 1 ml of binding buffer with (●——●) or without (○——○) the 10K-peptides (5 μg/ml) the times indicated and the fibronectin concentrations of the media were determined by radioimmunoassay. The protein concentrations of the cultures were 294 ± 19 μg. Abscissa: total fibronectin released (μg); Ordinate: time (hours).

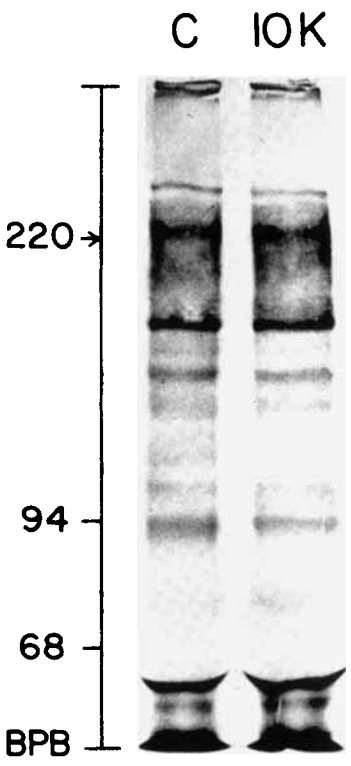

Fig. 4. SDS-polyacrylamide gel electrophoresis of the polypeptides released from cultured fibroblasts by 10K-peptides. Cultures of human lung fibroblasts were labeled with ^{35}S-methionine (50 μCi/ml) for 4 hours. The cells were then washed with serum-free media containing excess of unlabeled methionine and incubated in serum-free media plus 10K-peptides (5 μg/ml) for 3 hours. The polypeptides of the supernatants were precipitated with trichloracetic acid (10% final concentration), washed with acetone, and analyzed in a 5% gel slab with spacer. The gel was then subjected to autoradiography shown here. C: supernatant from control cells; 10K: supernatant from cells treated with 10K-peptides. An arrow indicates the position of purified plasma fibronectin run in parallel with the samples. Molecular weights of the marker proteins ($\times 10^3$) are given.

TABLE II. Effects of Some Hormonal Factors on the Fibronectin Production of Cultured Human Fibroblasts

Factor	Fibronectin in media (μg)
None	0.8
EGF (2 ng/ml)	0.6
Insulin (5 μg/ml)	0.8
Dexamethasone (50 ng/ml)	0.9
SGFs (20 μg/ml)	0.7
Rat 10K-MSA-Peptides (100 ng/ml)	0.7
10K-peptides from lung carcinoma cells (5 μg/ml)	0.8
10K-peptides (5 μg/ml)	3.3
10K-peptides (5 μg/ml) + PMSF (10 μg/ml)	0.7

Dense cultures of human lung fibroblast (60 mm diameter dishes) were washed with binding buffer and exposed to the hormones described in 3 ml of binding buffer for 3 hours. The fibronectin concentrations of the media were determined by radioimmunoassays. The protein concentrations of the cultures were 373 ± 17 μg.

peptides was apparently unaltered, suggesting that there may be a specific site close to the plasma membrane where the peptides act.

Virus-transformed and tumor-derived cell lines produce growth factors assumed to be important for their altered growth behavior [12, 17]. The sarcoma-derived peptide fractions described contain growth stimulating activities [14, 18]. Therefore, it was of value to try to correlate whether the release of fibronectin is linked to the MSA-like stimulation of growth. We tested sarcoma growth factors [24], rat MSA-peptides of the molecular weight of 10,000 [25], insulin, and epidermal growth factor in an analogous study. The 10,000 molecular weight fraction with growth promoting properties [Todaro et al, unpublished observations] from the supernatants of human lung carcinoma cells (9812), was used as a control. None of these peptides were able to cause a rapid release of fibronectin from cultured human fibroblasts into their media. Murine sarcoma growth factors, although able to reversibly transform murine cells [24], did not release fibronectin from human fibroblasts. We did not, however, test them in murine cell cultures, where they might be effective. The fibronectin-releasing activity produced by the fibrosarcoma cells is apparently unrelated to the growth promoting (MSA) peptides, but is a small molecular weight peptide that is copurified together with 10,000 molecular weight MSA-peptides. We have not succeeded in purifying these activities apart from each other thus far. It is not known whether the 10K-peptide(s) is also produced by other cell lines. This will be studied later after the further purification of the peptide(s).

The 10K-peptides may be related to plasminogen activators that are produced by transformed cells [15, 16]; however, the reported molecular weights of plasminogen activators produced by cultured fibroblasts are between 85,000–36,000 [26] which would suggest this activity is distinct from these factors. The ability of the 10K-peptide(s) to activate plasminogen has not been studied thus far. The role of plasminogen activators in the release of fibronectin is not clear. Removal of plasminogen from transformed cells does not restore their cell surface fibronectin [27]. Several proteases including trypsin [6, 28] and thrombin [7] release fibronectin from cells. Thrombin apparently attacks a specific site close to the membrane and releases fibronectin molecules that are apparently unaltered in size [7], whereas trypsin causes a significant cleavage [6, 28]. Transformed cells produce and release fibronectin into their media without detectable difference in size when compared to fibronectins produced by normal cells [29]. This does not exclude the possibility of the cleavage of a short polypeptide. Thus, fibronectins produced by transformed cells might have different affinities to the cellular skeletal actin-containing structures proposed to serve as inserting structures for cell surface fibronectin [30–32].

The 10K-peptide(s) described here is the first cell-derived factor found to release fibronectin. This peptide(s) may help sarcoma cells in destroying the extracellular matrix that contains fibronectin [33–36] and thus they may somehow be related to the infiltrating properties of fibrosarcoma cells.

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