CELL TO SUBSTRATUM ADHESION-PROMOTING ACTIVITY RELEASED BY NORMAL AND VIRUS-TRANSFORMED CELLS IN CULTURE

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ABSTRACT

It is demonstrated here that cultured fibroblasts release into their medium a nondialyzable, protease-sensitive factor(s) capable of promoting the adhesion and spreading of virus-transformed rat fibroblasts on a plastic substratum. A relatively sensitive biological assay is described for quantitation of the adhesion-promoting factor (APF) activity in serum-free, conditioned medium harvested from the cultures. Evidence is presented which indicates that the primary mode of action of the APF is by binding to and modifying the properties of the substratum.

Conditioned media harvested after 24 h of incubation in similarly populated cultures of normal fibroblasts of diverse animal species exhibited similar levels of APF activity. However, conditioned media obtained from Rous sarcoma virus (Prague strain)-transformed and avian sarcoma virus B77-transformed rat fibroblasts exhibited three- to sixfold lower levels of APF activity than media conditioned in parallel cultures of heterologous or homologous normal fibroblasts. Cultivation of B77 virus-transformed rat cells in the presence of dibutyl cyclic AMP and theophylline led to as much as a sevenfold increase in the level of APF activity appearing in the culture medium, with a concomitant increase in the adhesiveness of the cells to the culture substratum.

The results support the role of extracellular macromolecules in cell to substratum adhesion. It is postulated that the reduced adhesiveness of transformed cells to a substratum may be at least partially owing to a deficiency in the production and/or release of APF-like macromolecules.

The adhesion of cells to one another and to non-cellular substrata is thought to play an essential role in such diverse phenomena as morphogenetic movements, metastasis of tumors, and infiltrative movements of normal and malignant cells (10, 11, 27, 32, 36). In addition to their relevance to these forms of *in vivo* cell behavior, studies of cell adhesion are also pertinent to cell movement and to the morphology and growth patterns of cells in culture

(1, 7, 39, 40). Transformed cells are less adherent to the substratum than untransformed cells (13, 30), and this reduced interaction with the substratum may be related to abnormal properties such as loss of contact or density inhibition of growth (25).

In spite of intensive investigations, the basic mechanisms involved in cell adhesion are still largely unknown. Little is known concerning the actual molecules involved in cell adhesion or

about the derangements which may cause the altered adhesive properties of neoplastic cells. Glycoproteins or mucopolysaccharides may mediate the adhesive bond per se (4, 28) while, as suggested by recent studies, intracellular contractile elements (e.g., microfilaments) subjacent to the plasma membrane may be involved in the cell's spreading activity (40, 41). The adhesive behavior of cells in culture is also affected by extracellular macromolecules released by the cells into their environment. Some of these extracellular macromolecules are deposited by the cells upon the culture's substratum, rendering it more physicochemically acceptable to firm cell adhesion and spreading. Hence, Weiss (37) demonstrated that a glass surface was altered so that cells were more adhesive to it owing to materials deposited on the glass by cultured cells. Culp and Black (9) used chelating agents such as ethylenedinitrilo tetraacetic acid (EDTA) (for Mg^{2+} and Ca^{2+} chelation) or ethylenebis (oxyethylenenitrilo) tetraacetic acid (specifically for Ca^{2+} chelation) to remove 3T3 mouse fibroblasts or simian virus 40 (SV40)-transformed 3T3 cells from the culture substratum (glass or plastic). They found that the surface of the substratum, although free of any cells, vesicles, or organelles, was covered with a glycoprotein material. The amount of this substratum-attached material correlated with the flat, highly spread morphology of 3T3 cells and concanavalin A-selected revertant cells of the SV40-transformed cells, i.e. these flat, contact-inhibited cell lines deposited three to six times more of this material than the smaller, spindle-shaped transformed cells. In a related subsequent study, Culp (8) found that SV40-transformed cells attached to cover slips coated with glycoprotein deposits from either 3T3 or SV40-transformed 3T3 cells with a faster initial rate and to a higher saturation level than to untreated cover slips, whereas 3T3 and revertant SV40-3T3 cells showed no preference.

In addition to their detection in association with the culture substratum, cell-released macromolecules that affect cell adhesion or spreading have also been detected in the supernatant medium of cultured cells. Numerous studies have demonstrated that cultured cells release into their supernatant medium tissue-specific macromolecular factors which, when added to fresh suspensions of homologous cells, enhance cell reaggregation (22, 26). Takeichi (34) described the presence of a nondialyzable protease-sensitive factor in medium conditioned by mass cultures of chick embryo fi-

broblasts that enhanced the spreading of chick chondrocytes on plastic. Yasuda (22) described the presence of a nondialyzable, protease-sensitive factor in serum-free, chick embryo fibroblast-conditioned medium that promoted spreading of fibroblasts derived from chick embryo lungs. The precise chemical and cellular nature and detailed mechanisms of action of the cell-released factors which promote cell adhesion and spreading remain to be determined.

In extension of the above findings, the present work demonstrates the presence of a nondialyzable factor(s) in serum-free, fibroblast-conditioned medium (CM) that promotes the adhesion and spreading of virus-transformed rat cells on a plastic substratum. A simple and relatively sensitive biological assay has been devised for quantitation of this adhesion-promoting factor (APF) activity in CM. This assay system has been applied in partial characterization of the APF, in evaluating the effects of certain altered physiological states of cultured cells on the level of APF activity appearing in their culture medium, and in a comparative analysis of the levels of APF activity appearing in the culture media of various normal and virus-transformed cells.

MATERIALS AND METHODS

Cell Cultures

Primary cultures of chicken, mouse, and rat fibroblasts were prepared by trypsinization of the minced embryo body walls. 12-day old chicken, 8-10-day old mouse, and 14-16-day old rat embryos were used. Primary cultures of adult human fibroblasts were prepared by trypsinization of the outgrowth of surgically removed human skin biopsies. The R(B77) cell line (uncloned) was originated by infecting sparse cultures of rat embryo fibroblasts in their seventh passage with a concentrated preparation of B77 strain avian sarcoma virus under conditions similar to those described by Altaner and Temin (3). The R(B77) A cell line, a clonal derivative of the R(B77) cells, was isolated by the soft agar cloning technique (24). XC tumor cells, a gift of Dr. H. Temin, University of Wisconsin, were originally obtained from a rat sarcoma induced in newborn rats by Rous sarcoma tissue mince prepared from a Prague virus-induced chicken tumor (33). Except as otherwise specified in the individual experiments, all cell cultures were routinely grown in 90-mm plastic culture dishes in an environment of 5% CO_2 in air which was humidified and maintained at 37°C. The culture medium consisted of Eagle's minimal essential medium (MEM) supplemented with the indicated concentrations of calf or fetal calf serum and with 50 U/ml of penicillin and 50 μ g/ml of streptomycin. Routinely, the culture medium was supplemented with

5–10% calf serum for chicken and rat embryo fibroblasts, with 5–10% fetal calf serum for human fibroblasts and mouse embryo fibroblasts, and with 2.5–5% calf serum for R(B77), R(B77) A, and XC cells. When grown to confluence, the cultures were subcultured by the trypsinization procedure. The cells were routinely tested for and found to be free of mycoplasma contamination.

All three lines of virus-transformed cells display morphological and growth properties generally characteristic of transformed cells in culture. Both the R(B77) A and XC cells are distinguished from the R(B77) cells by their great adhesiveness to the culture substratum. This was indicated by a greater extent of attachment and spreading by the R(B77) A and XC cells after inoculation of the trypsin- or EDTA-dispersed cells into plastic or glass dishes containing serum-free medium, as well as a greater resistance of the adherent cells to the detachment action of trypsin, versene, or mechanical agitation.

Preparation of Conditioned Medium

In routine preparation of conditioned medium (CM) for analysis of APF activity, the following procedure was used. Healthy, confluent cultures of the various cells grown in 90-mm plastic culture dishes as described above were rinsed three times with 10-ml portions of Dulbecco's phosphate-buffered saline, pH 7.4, (PBS²⁺), and then supplied with 7-ml portions of serum-free MEM. After about 24 h of incubation at 37°C in a humidified 5% CO₂ incubator, the culture supernate, now termed CM, was harvested, centrifuged at 7,500 *g* for 30 min at 4°C to remove cell debris, and used immediately or stored at –20°C for later use. In some instances, different conditions were used in preparation of CM, and some CM preparations were dialyzed before analysis; such will be described, however, in the particular experiments. Dialysis of CM was performed at 4°C by use of cellulose tubing with an average pore radius of 24 Å. When dialysis was not aseptically performed, the CM sample was subsequently sterilized by filtration through a Millipore filter, pore size 0.45 μm (Millipore Corp., Bedford, Mass.). In order to prevent excessive loss of the APF activity of the CM, presumably by adsorption to the filter during filtration, the filter was pretreated for several hours with a 0.5% solution of BSA.

Determination of Protein

The amounts of cell and CM protein associated with the cultures examined were determined by the Lowry procedure (23). Crystalline bovine serum albumin was used as a standard. Before analysis for protein, the CM samples were filtered through a Millipore filter (pore size 0.45 μm) and dialyzed for 24 h against two changes of 1,000 vol 0.15 M NaCl.

Assay for APF Activity

Although fibroblast CM was effective in promoting both cell adhesion and spreading in a seemingly parallel

fashion, only the adhesion-promoting effect was quantitated in the present study. APF activity is defined by its capacity to promote cell adhesion to an appropriate substratum. The present assay system employs R(B77) cells as test cells for the APF activity and the surface of Falcon plastic tissue culture dishes (Falcon Plastics, Los Angeles, Calif.) as the substratum. Under the conditions of the standard assay described below only a small fraction of inoculated R(B77) cells become adherent to the culture substratum in the absence of added APF activity. However, in the presence of an adequate amount of APF activity as supplied by serum or serum-free fibroblast CM, most of the cells become adherent and spread. In addition to their requirement for added APF activity, the R(B77) cells offered the advantage of constituting an established cell line which displayed luxuriant growth in culture, thus allowing for maintenance of a continuous and abundant supply of test cells. Another important attribute of the R(B77) cells in terms of their feasibility for the APF activity assay is that they can tolerate serum-deprivation for at least 48 h without any significant effect on their viability. For the purpose of comparing the quantity of APF activity in various test samples, a unit of APF activity is defined as that amount of activity which leads to an increment of 15% cells adherent under the conditions of the assay described below.

Before testing for APF activity, the pH of the CM sample was adjusted by the addition of an appropriate amount of 0.15 N NaOH or 0.15 N HCl. The APF activity assay was conducted at pH 7.4 as follows. With MEM (serum-free) as a diluent, serial two-fold dilutions of test CM sample were prepared in glass tubes and 3-ml portions of the various dilutions were added to duplicate 60-mm plastic culture dishes. 3-ml portions of MEM were added to duplicate dishes to represent zero concentration of test sample. After addition of the medium each dish was supplied with 0.3 ml of a 2% solution of bovine serum albumin (fraction V) (BSA) prepared in MEM. 1 h after the addition of the BSA each dish was inoculated with 2.0×10^5 R(B77) cells suspended in 0.2 ml MEM. The cells were obtained by treatment of healthy confluent cultures grown in 90-mm dishes with a 0.05% solution of trypsin (Difco Laboratories, Detroit, Mich., 1:250) for 5–10 min. Trypsin activity was stopped by adding calf serum to a concentration of 10%. Before their addition to the dishes, the dispersed cells were thoroughly washed by repeated suspension in serum-free MEM and pelleting by centrifugation. After addition of the cells the dishes were rocked to distribute the cells uniformly and then transferred to a 37°C humidified CO₂ incubator. After 16 h of incubation the number of adherent cells in the cultures was determined. This involved rocking the dishes on a tray to suspend unattached cells which were then removed along with the culture supernate by aspiration. The adherent cells were removed from the dishes by incubation with 2 ml of versene solution (0.586 mM disodium versenate, 137 mM NaCl, 23.7 mM KCl, 8.1 mM Na₂HPO₄, and 0.137 mM KH₂PO₄ in distilled water) for 15–20 min followed by

gentle scraping of the dish surface with a rubber policeman. The detached cells were suspended in Ca^{2+} - and Mg^{2+} -free, phosphate-buffered saline, pH 7.4 (PBS), counted with a Coulter counter (Coulter Instruments, Inc., Hialeah, Fla.), and the averages of percent cells adherent in the duplicate dishes were determined. The amount of cell adhesion in the dishes containing zero concentration of test sample was termed background cell adhesion. This value, which was less than 5%, was subtracted from the averages of percent cell adherent in the duplicate dishes containing the varying concentration of test sample. The values of cell adhesion corrected for background were plotted against the percent (vol/vol) of test sample on a linear scale, and the units of APF activity in the 3-ml assay volume of sample were approximated from the resultant dose-response curve. (The number of units of APF activity/3 ml of undiluted sample equals $100\% \div$ percent sample concentration corresponding to 15% cells adherent.) Typical dose-response curves are presented in Results. In the present study, the APF activity assay was used only in comparing the quantity of activity in CM preparations simultaneously assayed. In replicate assays on portions of a given sample of CM, the units of APF activity determined showed a standard deviation of no greater than 10%.

Materials

Materials were purchased from the following sources: MEM, MEM amino acids, MEM vitamins, calf and fetal calf sera from Grand Island Biological Company, Grand Island, N. Y.; penicillin and streptomycin from E. R. Squibb & Son, New York; 60- and 90-mm plastic tissue culture dishes from Falcon Plastics, Los Angeles, Calif.; trypsin 1:250 from Difco Laboratories, Detroit, Mich.; trypsin (twice crystallized), soybean trypsin inhibitor, DNase, RNase, cycloheximide, bovine serum albumin (fraction V), and crystalline bovine serum albumin from Sigma Chemical Company, St. Louis, Mo.; N^6 , O^2 -dibutyl adenosine -3' -5'cyclic phosphate, A grade, and theophylline, B grade, from Calbiochem Corp., La Jolla, Calif.

RESULTS

Characteristics of APF Activity Assay

To demonstrate the effect of BSA in the APF activity assay, typical dose response curves resulting from conducting the assay on rat fibroblast-CM with and without the addition of BSA are presented in Fig. 1 A. (Fig. 1 B will be discussed below.) In the absence of BSA, background cell adhesion was about 32%. This level of cell adhesion remained unchanged as the CM concentration was increased up to about 6%; beyond this value, the extent of cell adhesion increased progressively as the CM concentration was increased. By adding 0.2% BSA to the assay dishes, back-

FIGURE 1 Adhesion of R(B77) cells in dishes containing varying concentrations of rat fibroblast CM with (●) or without BSA (○) (A); and in dishes whose surfaces were pretreated with varying concentrations of rat fibroblast CM with (●) or without BSA (○) (B). The experimental procedures for A were as described for the APF activity assay (see Materials and Methods) except that BSA was excluded from a portion of the dishes. In B, the surfaces of 60-mm plastic culture dishes were pretreated by adding 3-ml aliquots of the indicated concentrations of CM (portions of same preparations used in A) followed by the addition of 0.3 ml of 2% BSA (V) to a portion of the dishes. After 16 h incubation at 4°C the CM was removed from the dishes, and each dish was rinsed twice with 5-ml portions of PBS, supplied with 3 ml of fresh MEM, and then inoculated with 2.0×10^5 R(B77) cells. The dishes of A and B were inoculated with cells simultaneously and incubated for 16 h, after which time the adherent cells in the dishes were enumerated. The data are from a representative experiment. The averages and ranges of the percent cells adherent in triplicate dishes are presented.

ground cell adhesion was reduced from 32% to 2%. Against this reduced level of background cell adhesion, increased cell adhesion can be noted at substantially lower concentrations of CM. In terms of the concentration of CM which gave rise to an increment of 15% cells adherent (i.e. contained 1 U of APF activity), the use of BSA led to about a sixfold increase in the sensitivity of the assay. The dose response curves with BSA ap-

peared sigmoidal in shape and showed a rather sharp increase in cell adhesion with increased CM concentrations up to about 10%; above this value, the slope of the curve was considerably reduced and a plateau level of cell adhesion was reached at 50% CM. It should be noted that at concentrations in excess of 10% CM the adhesion-promoting effect of the CM was significantly enhanced by the presence of BSA. The basis for this phenomenon and for the sigmoidicity of the dose response curves has not been determined. Similar results were obtained when the R(B77) cells used were obtained from parent cultures dispersed with the aid of versene rather than with trypsin.

Both BSA and fibroblast-CM were capable of exerting their respective effects on cell adhesion when used in pretreating the dish surface. To demonstrate this phenomenon, the following experiment was performed. A portion of the R(B77) cell suspension used in the experiment of Fig. 1 A was simultaneously inoculated into dishes whose surfaces were pretreated for 16 h with varying concentrations of rat fibroblast CM with or without BSA. Before addition of the cells, the pretreated dishes were thoroughly washed with PBS and then supplied with fresh MEM. The resultant dose response curves showing cell adhesion as a function of the amount of CM used in pretreating the dish surface are presented in Fig. 1 B. It can be seen that these curves are similar to the corresponding curves of Fig. 1 A which show cell adhesion as a function of the amount of CM in the culture. Preincubation of suspended R(B77) cells in fibroblast CM for 16 h followed by washing of the cells and inoculating them into serum-free medium failed to stimulate increased cell adhesion. Likewise, preincubation of the cells in 0.2% BSA failed to reduce the extent of cell adhesion when the cells were inoculated into serum-free medium. These results taken together most probably indicate that both the APF and BSA exert their respective effects on cell adhesion by binding to and modifying the properties of the substratum.

Fig. 2 shows photomicrographs illustrating the appearance of R(B77) cells 16 h after their inoculation into plastic culture dishes containing MEM alone, MEM with 0.2% BSA, rat fibroblast CM, and MEM with 2.5% calf serum. Of the fraction of the cells which become adherent in the dishes with MEM alone (Fig. 2a), most retain a spherical, unspread shape. In the dish containing MEM plus BSA (Fig. 2b) where less than 5% of the cells are adherent, the unattached cells tend to form relatively large aggregates over a period of several

hours owing to their mutual stickiness. In dishes containing rat fibroblast CM (Fig. 2c) or calf serum (Fig. 2d), most of the cells are adherent and spread. The R(B77) cells exhibited a similar appearance in the presence of adequate concentrations of other sera or CM from cultures of other cell types.

Fig. 3 shows typical dose response curves resulting from conducting the APF activity assay on CM preparations from cultures of various normal and virus-transformed cells. It can be seen that all the resultant dose response curves are somewhat sigmoidal in shape, with only those corresponding to the CM preparations from the normal fibroblast cultures showing plateau levels of cell adhesion. These plateau levels of cell adhesion were quite similar in value. It can be seen that the 15% cells adherent value chosen to correspond to 1 U of APF falls within the initial rapidly rising portions of the plotted dose response curves.

Properties of the APF in Fibroblast-CM

Some physical and chemical properties of the APF in CM obtained from cultures of rat, chicken, and human fibroblasts were investigated. The APF of all three sources failed to pass through a cellulose dialysis membrane, indicating that it is nondialyzable and thus most probably a macromolecule. As noted in Table I, when CM from rat or human fibroblast cultures was heated at 65°C for 10–60 min or at 100°C for 10 min, most or all of the APF activity was destroyed. Although the APF activity in chick fibroblast CM was found to be more heat resistant than that in human fibroblast and rat fibroblast CM, most of the activity in the former preparation was inactivated when heated at 65°C for 60 min or at 100°C for 10 min. The effect of various enzymes on the APF activity in the CM preparations was evaluated. As shown in Table II, treatment of either CM preparation with the indicated concentration of trypsin or chymotrypsin for 30 min at 37°C resulted in complete destruction of the APF activity. Treatment of the CM preparations with either of these proteolytic enzymes in the presence of soybean trypsin inhibitor resulted in essentially no destruction of APF activity, indicating that catalytically active enzymes are required. Treatment of the CM preparations with DNase or RNase for 3 h at 37°C had no appreciable effect on the APF activity. These results indicate that the APF is most likely protein in nature.

In order to determine the requirement for divalent cations for expression of its constituent APF

FIGURE 2 Phase-contrast micrographs of R(B77) cells 16 h after inoculation into plastic culture dishes containing: (a) MEM alone; (b) MEM with 0.2% BSA; (c) rat fibroblast CM; and (d) MEM with 2.5% calf serum. Original magnification $\times 100$. Bar equals $100\ \mu\text{m}$.

FIGURE 3 Dose-response curves obtained in APF activity assay on CM preparations from confluent cultures of normal mouse embryo fibroblasts (○), normal rat embryo fibroblasts (●), normal human fibroblasts (■), normal chicken embryo fibroblasts (▲), R(B77) cells (◇), R(B77) A cells (△), and XC cells (□). The various cultures were prepared and grown as described in the legend of Table V. The experimental procedures for preparation of the CM and for performing the APF

activity, portions of dialyzed CM without divalent cations and with Mg^{2+} and/or Ca^{2+} were tested for the ability to promote the adhesion of R(B77) cells under conditions similar to those of the APF activity assay. The results of performing this test on serum-free CM from cultures of chick embryo fibroblasts are presented in Table III. Similar results were obtained when CM preparations from cultures of other cell types were used. While leading to 75% cell adhesion in the presence of the indicated concentrations of Mg^{2+} and Ca^{2+} , the CM failed to promote significant cell adhesion above the background value of 3% when divalent cations were absent. Although Mg^{2+} used alone was more effective than Ca^{2+} used alone, both

activity assay were as described in Materials and Methods. The CM samples analyzed were obtained from cultures which contained similar amounts of cell protein (1.8–2.3 mg/culture). The data are from a representative experiment. Each value is an average of the percent of cells adherent in duplicate dishes.

TABLE I
Effect of Heating or Boiling for Various Lengths of Time on APF Activity in CM Preparations from Cultures of Various Fibroblasts*

Treatment	APF activity‡		
	RF CM§	CF CM	HF CM
	%	%	%
None (control)	100	100	100
65°C for 10 min	15	50	6
65°C for 30 min	9	30	<6
65°C for 60 min	3	23	0
100°C for 10 min	0	12	0

* 7-ml portions of rat fibroblast (RF) CM, chick fibroblast (CF) CM, and human fibroblast (HF) CM were treated as indicated. The control and heat-treated samples were dialyzed against 100 vol of fresh MEM and then assayed for APF activity as described in Materials and Methods.

‡ Expressed as percentage of APF activity present in the corresponding control samples. The control samples of RF CM, CF CM, and HF CM, contained 80, 65, and 57 U of APF activity, respectively.

§ Abbreviations explained above.

divalent cations had to be present in order to obtain maximal induction of cell adhesion by CM. Based upon the trypan blue dye exclusion test, the number of dead cells in the dishes containing CM with Mg^{2+} and Ca^{2+} and in those containing CM without divalent cations were 5% and 30%, respectively, after 4 h of incubation. The majority of the unstained, apparently live cells of the cultures with CM minus divalent cations proceeded to attach and spread, however, when supplied with adequate amounts of Mg^{2+} and Ca^{2+} . Similar divalent cation requirements were shown in order to obtain the induction of R(B77) cell adhesion in dishes whose surfaces were pretreated with CM but which contained APF activity-free medium.

All of the CM preparations tested were unable to induce the adhesion of Formalin-fixed R(B77) cells or cells incubated at 4°C under otherwise standard conditions of the APF activity assay.

Time-Course of Accumulation of APF Activity in Medium of Rat Fibroblast Cultures

Fig. 4 shows the time course of accumulation of APF activity in the medium of confluent rat fibroblast cultures over a 72-h time span. A measurable quantity of APF activity appeared in the culture medium as early as 4 h after addition of fresh medium. The level of APF activity increased pro-

gressively over the 72-h period of observation. Over the 72-h period of incubation in serum-free medium about 8% of the cells became detached from the cell monolayers. On the basis of the trypan blue dye exclusion test, essentially none of the adherent cells in the monolayers but most of the detached cells were dead. Results presented immediately below, suggesting a requirement for active cellular processes for release of the APF, render it highly unlikely that a significant portion of the APF activity is derived from dead cells.

Effect of Incubation at Low Temperature or with Cycloheximide on Level of APF Activity Accumulating in Medium of Rat Fibroblast Cultures

As shown in Table IV, the accumulation of APF activity in the culture medium was reduced to a

TABLE II
Effect of Various Enzymes on APF Activity in CM Preparations from Cultures of Various Fibroblasts*

Addition	APF activity‡		
	RF CM	HF CM	CF CM
	%	%	%
None (control)	100	100	100
0.01% trypsin	0	0	0
0.01% trypsin + 0.05% STI	103	95	100
0.001% chymotrypsin	0	0	0
0.001% chymotrypsin + 0.05% STI	87	83	90
0.01% DNase	110	105	97
0.01% RNase	90	96	93

* Solutions of the indicated enzymes or enzymes plus inhibitor were prepared in PBS^{2+} at 10 times the indicated concentrations, and 0.7-ml portions were added to 6.3-ml portions of indicated CM preparations. 0.7 ml of PBS^{2+} was added to 6.3 ml of each CM preparation to serve as controls. The samples containing trypsin or chymotrypsin or either of these enzymes plus soybean trypsin inhibitor (STI) were incubated for 30 min at 37°C. STI was then added to a concentration of 0.05% to the CM samples incubated with trypsin or chymotrypsin alone. The control samples and those to which DNase and RNase were added were incubated for 3 h at 37°C, after which time all samples were assayed for APF activity (see Materials and Methods). The data presented are from a representative experiment.

‡ Expressed as percentage of APF activity present in corresponding control samples. The control samples of RF CM, HF CM, and CF CM contained 66, 62, and 80 U of APF activity, respectively.

TABLE III
Demonstration of Divalent Cation-Requirement for
Expression of APF Activity in Conditioned Me-
dium*

Medium tested	Cells adherent† %
Dialyzed CM with 1 mM Mg ²⁺ + 1 mM Ca ²⁺	75.1 ± 4.0
Dialyzed CM with 1 mM Mg ²⁺	63.0 ± 3.6
Dialyzed CM with 2 mM Mg ²⁺	61.0 ± 3.7
Dialyzed CM with 1 mM Ca ²⁺	40.0 ± 3.2
Dialyzed CM with 2 mM Ca ²⁺	39.4 ± 2.7
Dialyzed CM without Mg ²⁺ and Ca ²⁺	3.2 ± 0.6
MEM with 1 mM Mg ²⁺ + 1 mM Ca ²⁺ ‡	3.0 ± 0.6

* Serum-free CM obtained from confluent cultures of chick embryo fibroblasts was dialyzed thoroughly against Mg²⁺- and Ca²⁺-free MEM (serum-free) to remove divalent cations from the original sample. 3-ml amounts of the dialyzed CM without divalent cations, and with the indicated concentrations of Mg²⁺ and/or Ca²⁺ (chloride salts) were added to triplicate 60-mm plastic culture dishes. 3-ml amounts of serum-free MEM were added to triplicate dishes to serve as an APF activity-free control medium. After the addition of the medium, the dishes were supplied with 0.3-ml amounts of a 2% BSA solution prepared in a medium identical in composition to that present in the respective dishes. 1 h after the addition of BSA, each dish was inoculated with 2.0×10^5 R(B77) cells obtained from trypsin-dispersed cultures. 4 h after addition of the cell inocula, the number of adherent cells in the cultures were determined. The procedures for preparation of the cell inocula and for determination of the number of adherent cells were as described for the APF activity assay in Materials and Methods.

‡ The values presented are the means and standard deviations of triplicate determination.

§ Control (APF activity-free) medium in which the extent of cell adhesion is referred to as background cell adhesion.

negligible level by incubation of the cultures at 4°C. Incubation of the cultures in the presence of 5 µg/ml of cycloheximide, an inhibitor of protein synthesis, led to a 75% reduction in the level of APF activity appearing in the culture medium over a 16-h period. On the basis of its effect on the incorporation of [¹⁴C]leucine into TCA-insoluble cellular material, the level of cycloheximide used led to about a 75% reduction in the rate of protein synthesis within 3 h after its addition to the cultures containing serum-free MEM. Incubation of the cultures at 4°C or with cycloheximide for the indicated time period did not significantly affect the viability of the cells. This was based upon the trypan blue dye exclusion test and the ability of

the trypsin-dispersed cells of the cultures to attach and spread properly when inoculated into fresh dishes containing MEM plus serum.

These results suggest that the APF is released by the cells into their culture medium as a result of active physiological processes, rather than being passively leaked or shed from the cells. These active processes appear to involve protein synthesis, at least in the case of rat fibroblasts.

Comparison of Levels of APF Activity Appearing in Culture Media of Various Normal and Virus-Transformed Cells

A comparison was made of the levels of APF activity appearing over a 24-h period in the media (serum-free) of parallel cultures of various normal and virus-transformed cells containing similar amounts of cell protein. Typical results from this comparative study are summarized in Table V. (See Fig. 3 for typical dose response curves obtained in conducting the APF activity assay on CM preparations from the various cultures.) Expressed as units/milligram of cell protein, the levels of APF activity accumulating in the media of the various normal cultures were quite similar. Interestingly, however, the levels of APF activity (units/milligram of cell protein) accumulating in the media of the virus-transformed cultures were three- to sixfold lower than those for the normal

FIGURE 4 Time-course of accumulation of APF activity in medium of rat fibroblast cultures. 7-ml portions of MEM (serum-free) were conditioned for the indicated periods of time in parallel confluent cultures of normal rat embryo fibroblasts (approximately 8.0×10^5 cells per 90-mm culture dish). The APF activity in the harvested CM samples was assayed as described in Materials and Methods. The data are from a representative experiment. Each point represents the average of determinations made on duplicate CM samples from replicate cultures. The duplicate values varied by no greater than 13%.

TABLE IV
Effect of Low Temperature and Cycloheximide on Accumulation of APF Activity in Medium of Rat Fibroblast Cultures*

Experiment	Incubation temperature	Addition	APF activity	
			U	%
1	36°C	None	50	100
	4°C	None	<3	<6
2	37°C	None	40	100
	37°C	Cycloheximide (5 µg/ml)	10	25

* In both experiments 7-ml portions of MEM (serum-free) were conditioned for 16 h under the indicated conditions in 7-day old parallel confluent cultures of rat embryo fibroblasts (approximately 8.0×10^6 cells per 90-mm culture dish). The cultures incubated at 4°C were prechilled at this temperature for 30 min before the addition of the fresh medium for conditioning. The cultures with cycloheximide were preincubated in the presence of 5 µg/ml of this additive for 3 h before addition of fresh medium (with or without cycloheximide) for conditioning. The harvested CM samples of experiment 2 were dialyzed against 4 liters of distilled water for 24 h and reconstituted by the addition of 0.1 vol of $10 \times$ concentrated MEM. (The small residual levels of cycloheximide remaining in the dialyzed CM samples had no effect on the adhesion of the R(B77) cells in the APF activity assay.) The APF activity in the CM samples was assayed as described in Materials and Methods. The data are from representative experiments. Each value is an average of determinations made on duplicate CM samples from replicate cultures. For both experiments, the duplicate values varied by no greater than 13%.

cultures. The media of both XC and R(B77) cultures exhibited nearly twice as much APF activity per milligram of cell protein as the medium of the less adhesive R(B77) cells. The specific APF activity values (expressed as units/milligram of CM protein) for the media conditioned in the various cultures varied considerably. However, those values for the conditioned media from the normal cultures were significantly greater than those for the conditioned media from the transformed cultures.

The decreased levels of APF activity demonstrable in the conditioned media from the transformed cultures were not associated with the presence of APF activity-inhibitory substances in these preparations which were detectable in experiments in which APF activity assays were per-

formed on mixtures of normal rat fibroblast CM and CM freshly harvested from cultures of XC or R(B77) cells (Table VI).

Effect of Preincubation of R(B77) Cell Cultures with Dibutyryl (But₂) Cyclic AMP and Theophylline on Level of APF Activity Appearing in Culture Medium

Treatment of transformed cells with But₂-cyclic AMP (a cyclic AMP analogue) or with the phosphodiesterase inhibitor, theophylline, has been shown to restore many of their abnormal properties to or toward normal (15-18, 31), including increasing their adherence to a substratum (19). It was of interest, therefore, to determine whether the relatively low levels of APF activity appearing in the medium of virus-transformed rat cell cul-

TABLE V
Comparison of Levels of APF Activity Accumulating in Culture Media of Normal and Virus-Transformed Cells*

Culture type	APF activity	
	Cell protein	CM protein
	U/mg	U/mg
Normal mouse fibroblasts	28.7	272.3
Normal rat fibroblasts	30.5	316.7
Normal chick fibroblasts	31.6	391.2
Normal human fibroblasts	27.4	466.0
R(B77) cells (transformed)	5.0	71.4
R(B77) A cells (transformed)	8.7	145.0
XC tumor cells (transformed)	9.8	200.0

* The transformed cultures were prepared by inoculating $1.0-3.0 \times 10^6$ cells per dish. Cultures of the remaining cell types were prepared by inoculating $2.5-5.0 \times 10^6$ cells per dish. All cultures were grown in 90-mm culture dishes containing 10-ml portions of MEM with 8% fetal bovine serum. After 4 days of incubation, the cultures were washed with PBS²⁺ and supplied with 7-ml portions of MEM (serum-free) for conditioning. After 24 h of incubation, the CM was harvested and the amount of cell protein in each culture determined. The CM samples from confluent cultures which were found to contain similar amounts of cell protein (1.9-2.3 mg per culture) were analyzed for protein content and assayed for APF activity as described in Materials and Methods. The total amounts of protein in the CM samples ranged from 0.1 to 0.2 mg. The data are from a representative experiment. Each value is an average of determinations made on two replicate cultures. The duplicate values varied by no greater than 15%.

TABLE VI

*Test of CM Preparations from Cultures of XC and R(B77) Cells for Presence of Inhibitor of APF Activity**

CM preparation	APF activity
	U
RF-CM (1 vol) + MEM (1 vol)	46.5
R(B77)-CM (1 vol) + MEM (1 vol)	11.7
XC-CM (1 vol) + MEM (1 vol)	10.7
RF-CM (1 vol) + R(B77)-CM (1 vol)	52.0
RF-CM (1 vol) + XC-CM (1 vol)	51.0

* 7-ml amounts of the indicated CM preparations and mixtures thereof were incubated at 37°C for 1 h and then assayed for APF activity as described in Materials and Methods. Data are from a representative experiment.

tures could be increased by treatment of the cells with But₂-cyclic AMP and theophylline. Table VII shows the effect of preincubation of R(B77) cell cultures for 3 days in the presence of But₂-cyclic AMP and/or theophylline on the subsequent level of APF activity (units/milligram of cell protein) appearing in the culture medium over a 24-h period. The specific APF activity values (units/milligram of CM protein) for the media conditioned in the control and drug-treated cultures are also presented. The indicated concentrations of But₂-cyclic AMP and theophylline used in combination led to a sevenfold increase in the units of APF activity/milligram of cell protein and a fourfold increase in the specific APF activity of the R(B77) cell CM. Theophylline used alone led to a threefold increase in both the units of APF activity/milligram of cell protein and the specific APF activity of the R(B77) cell-CM, whereas But₂-cyclic AMP used alone led to only slight increases in both the units of APF activity/milligram of cell protein and the specific APF activity of the CM. Similar results were obtained when the CM preparations were dialyzed against fresh serum-free medium before being assayed for APF activity. In other experiments it was found that incubation of cultured normal rat embryo fibroblasts with But₂-cyclic AMP and theophylline had no significant effect on the level of APF activity appearing in their culture medium.

DISCUSSION

The present work demonstrates the presence of a factor(s) in fibroblast CM that promotes the adhesion and spreading of virus-transformed rat fibro-

TABLE VII

*Effect of Preincubation of Cultured R(B77) Cells with But₂-cyclic AMP and Theophylline on Level of APF Activity Accumulating in the Culture Medium**

Addition to culture‡	APF activity	
	Cell protein (fold increase)	CM protein (fold increase)
	U/mg	U/mg
None (control)	3.6 (1)	39.4 (1)
But ₂ -cyclic AMP (0.25 mM) + theophylline (1 mM)	26.2 (7.3)	163.7 (4.1)
Theophylline (1 mM)	11.5 (3.2)	116.3 (2.9)
But ₂ -cyclic AMP (0.25 mM)	5.1 (1.4)	54.3 (1.3)

* Cultures of R(B77) cells were prepared in 90-mm culture dishes containing MEM + 5% calf serum by inoculating 1.5×10^6 or 4.0×10^6 cells per dish. On the following day, duplicate cultures which were inoculated at 4.0×10^6 cells per dish were supplied with the indicated concentrations of But₂-cyclic AMP + theophylline, or theophylline alone. Duplicate cultures which were inoculated at 1.5×10^6 cells per dish were supplied with the indicated concentration of But₂-cyclic AMP or with no additive (control). After incubation of the cultures for 3 days in the presence of the additives, the latter were removed, the cultures were washed with PBS²⁺, and then supplied with 7-ml portions of fresh, serum-free MEM (without additives) for conditioning. After 24 h of conditioning, the culture medium was harvested and the amount of cell protein in each culture determined. The CM preparations were analyzed for protein content and assayed for APF activity as described in Materials and Methods. At the time of harvest of the CM, the amount of cell protein in the variously treated cultures ranged from 2.2 to 2.6 mg per dish. The total amounts of protein in the various CM preparations ranged from 0.17 to 0.24 mg. Data are from a representative experiment. Each value is an average of determinations made on two replicate cultures. The duplicate values varied by no greater than 15%.

‡ Incubation of R(B77) cells with the indicated concentrations of But₂-cyclic AMP + theophylline or theophylline alone was found to exert a marked inhibitory effect on the growth of the cells, while But₂-cyclic AMP used alone had little or no effect in this regard (unpublished observations). Hence, in an effort to obtain similarly populated cultures for use in preparation of CM following 3 days of incubation, the cultures to which the But₂-cyclic AMP + theophylline or theophylline alone were added contained a greater number of cells than those supplied with no additive (controls) or with But₂-cyclic AMP.

blasts upon a plastic substratum. Varying amounts of CM promoted the adhesion and spreading of R(B77) cells in a seemingly parallel fashion, suggesting that the same factor(s) may be responsible for both effects. Since the effect of CM on cell adhesion can be more conveniently and accurately quantitated than its effect on cell spreading, it was decided that only the adhesion-promoting effect would be analyzed in the present work. A relatively sensitive biological assay is described for estimation of the adhesion-promoting factor (APF) activity in CM. This assay used R(B77) cells as test cells for APF activity. The assay has been applied in partial characterization of the APF, in evaluating the effects of certain altered physiological states of cultured cells on the level of APF activity appearing in their culture medium and in a comparative analysis of the levels of APF activity appearing in the media of cultures of various normal and virus-transformed cells.

The APF activity of CM was not species specific, as CM preparations from similarly populated cultures of rat, chicken, mouse, and human fibroblasts exhibited similar levels of APF activity when tested on R(B77) cells. The APF activity in the various CM preparations examined was found to be nondialyzable, heat sensitive, and sensitive to proteolytic enzymes, suggesting that it is most probably protein in nature.

In terms of its effect on R(B77) cells, two possible primary modes of action of the APF were considered. One is that it adsorbed on the surface of the plastic culture dish and rendered it more physicochemically acceptable to firm cell adhesion and spreading. Another is that it induced some physiological or structural alteration in the cells themselves which enhanced their adhesive capacity. Contrary to the latter possibility, it was found that preincubation of suspended R(B77) cells with CM failed to enhance significantly the adhesion of the cells when they were inoculated in dishes containing serum-free medium. In accord with the former possibility, however, the adhesion of the cells was promoted by pretreating the dish surface with CM. Moreover, the dose response curves depicting the extent of cell adhesion in dishes containing varying amounts of CM were similar to those showing the extent of cell adhesion in dishes pretreated with corresponding amounts of CM. Hence, it would appear that the APF mediates its effect by binding to and modifying the properties of the dish surface. The inability of CM to promote the adhesion of R(B77) cells incubated at

4°C suggests that the expression of the APF activity required some active cell process(es). Although other explanations are possible, this active process may involve undulation of the cell membrane or protrusion of pseudopodia needed to form effective contact with the substratum (35). The requirement for divalent cations (Ca^{2+} and Mg^{2+}) may be partially owing to a need for their participation in such processes. The divalent cations may also be directly involved in formation of the adhesive bond per se between the cell surface and substratum. The latter possibility is consistent with the ability of versene, a divalent cation chelator, to detach CM-induced adherent R(B77) cells. Since the presently described APF is produced by a variety of cell types and appears to function in a substratum-bound state, it may possibly be analogous to the adhesion-enhancement constituent(s) of the substratum-associated cellular microexudates demonstrated in other cell systems (8, 9, 37). When one considers its apparent affinity for the plastic substratum, some of the APF is probably deposited directly on the substratum by the cells and some is most probably deposited from the pool of medium-secreted APF. With the availability of a biological assay for measuring its activity, the accumulation of APF in the medium of cultured cells represents a convenient source of the factor for isolation and characterization, and constitutes a highly manipulatable system for investigating the cellular physiological processes affecting release of the factor.

Experiments are presented which demonstrate the effectiveness of low temperature or cycloheximide in inhibiting the accumulation of APF activity in the medium of rat fibroblast cultures. These results suggest that the APF is released by the cells as a result of active physiological processes, apparently involving protein synthesis, rather than by passive leakage. Since cultured cells have been shown to release surface macromolecules into the medium via what appears to be a natural and continuous process (20), it is tempting to speculate that the APF described here may be a shed cell surface component.

In comparison with homologous or heterologous normal fibroblasts, the lines of virus-transformed rat fibroblasts examined here showed severalfold lower levels of APF activity appearing in their culture media after a constant incubation period. The decreased levels of APF activity demonstrable in the conditioned media from the transformed cultures were not associated with the

presence of APF activity-inhibitory substances in these preparations which were detectable in experiments in which APF activity assays were performed on mixtures of normal rat fibroblast CM and CM from cultures of XC or R(B77) cells. The reduced levels of APF activity appearing in the media of the transformed cultures are correlated with reduced adhesiveness of these cells to the culture substratum relative to normal rat fibroblasts. This is indicated by a reduced extent of adhesion and/or spreading by the transformed cells, particularly when inoculated into dishes containing serum-free medium, as well as by a greater sensitivity of the transformed cells to the detachment action of trypsin or versene (unpublished observations).

The level of cyclic AMP in fibroblasts appears to be important in the control of cell shape (15, 17, 31), growth (15, 18, 31), motility (17), and adhesiveness to a substratum (19, 38). The rapid growth and low adhesiveness of some transformed cells appear to be related to their reduced levels of cyclic AMP. Treatment of transformed cells with But₂-cyclic AMP (a cyclic AMP analogue) or the phosphodiesterase inhibitor, theophylline, has been shown to restore many of their abnormal properties to or towards normal (15-19, 31), including increasing their adherence to a substratum. Results are presented here which show that preincubation of R(B77) cells with But₂-cyclic AMP and theophylline led to a severalfold increase in the level of APF activity appearing in their culture medium. Although less effective than when used in combination with But₂-cyclic AMP, theophylline used alone also led to a substantial increase in the level of APF activity appearing in the medium of the R(B77) cell cultures, while But₂-cyclic AMP used alone led to only a marginal increase. Theophylline presumably leads to increased levels of intracellular cyclic AMP by inhibiting the cellular phosphodiesterase which degrades cyclic nucleotides. These results suggest that the production and/or release of APF activity is influenced by the level of cellular cyclic AMP. Accompanying the increased levels of APF activity induced by But₂-cyclic AMP and theophylline, the treated transformed cells became more flattened or spread upon the culture substratum, exhibited greater resistance to the detachment action of trypsin or versene, and showed a greater extent of adhesion and spreading when trypsin dispersed and inoculated into fresh dishes containing serum-free medium (unpublished observations). It is con-

sidered highly likely that the increased adhesiveness of the drug-treated cells as indicated by the above parameters was at least partially owing to increased release of APF activity by the cells.

Alteration in the intrinsic physicochemical properties of the cell membrane known to accompany neoplastic transformation of cells (2, 5, 6) undoubtedly contributes toward the altered adhesive behavior of the transformed cells. The results presented here and elsewhere indicate that cultured cells produce extracellular macromolecules which function in their adhesion to a solid substratum, perhaps by acting as a type of "glue." A deficiency in the production and/or release of these adhesion-related macromolecules may contribute toward the diminished adhesive capacity exhibited by transformed or neoplastic cells. Experiments are in progress in this laboratory to determine whether other lines of transformed cells show reduced levels of APF activity appearing in their culture media relative to cultures of their untransformed counterparts. If the generality of this phenomenon can be established, such may constitute a useful phenotypic distinction between normal and transformed cells in culture and may aid in the explanation of their differential adhesiveness to a solid substratum.

As did fibroblast CM, serum was also found to contain a nondialyzable, protease-sensitive factor(s) that promoted the adhesion and spreading of virus-transformed rat cells. The assay used here for quantitation of APF activity in CM can also be effectively applied in the quantitation of the APF activity in serum (unpublished findings). The serum component(s) required for the adhesion of the R(B77) cells examined here and for other types of cells described elsewhere (12, 21) may be similar to the above-discussed APF secreted by cultured cells. This notion is consistent with the finding that cultured cells released serum-like protein components into their medium (14, 29). The inability of certain types of cells to adhere properly in the absence of serum may result, at least in part, from a deficiency in the production of essential adhesion-related macromolecules (e.g., APF), hence requiring that they be exogenously supplied by serum or CM. The presence of such macromolecules in serum may be partially derived from the interstitial fluids bathing connective tissue being endogenously synthesized by the constituent fibroblasts. In this laboratory it was found that both serum and serum-free fibroblast CM contain nondialyzable, protease-sensitive factors capable of

promoting the adhesion and spreading of Rous sarcoma virus-transformed rat cells upon collagen gel substrata (unpublished observations). Further work is required to determine the specific nature and possible identity of the factors operative on plastic and collagen gel substrata. Since collagen is the predominant matrix material for anchorage and support of fibroblasts in connective tissue, the cell-released factor that promotes adhesion upon it *in vitro* may also play an important role in cell to substratum adhesion interactions occurring in tissues *in vivo*.

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