A Factor from HeLa Cells Promoting Colonial Growth of Human Fibroblast-like Cells in Culture*

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SUMMARY

Irradiated HeLa cells, lysed HeLa cells, and medium from irradiated HeLa cell cultures have been used as sources of a substance stimulating colonial growth of fibroblast-like cells derived from human amnion cultures. Preliminary characterization suggested that the stimulating substance was not dialyzable, was inactivated by acid or alkaline hydrolysis, was resistant to degradation with deoxyribonuclease and ribonuclease, and was susceptible to tryptic digestion but resistant to boiling.

Interactions between animal cells of diverse cytologic type are of interest as regards the possible relation to in vivo phenomena underlying the organization of tissues and host cellular response to malignant growth. A growth factor for human fibroblast-like cells in culture elaborated by HeLa cells was discovered in the course of attempts to propagate human amnion cells by the use of irradiated HeLa "feeder" layers (8). In several experiments, irradiated HeLa cells were added to amnion cell monolayers; these cultures were overgrown by fibroblast-like cells days to weeks earlier than were control amnion cultures without HeLa cells. This paper presents evidence for existence of a growth-stimulating substance released by HeLa cells and describes some properties of the factor.

MATERIALS AND METHODS

Media.—Solutions used as previously described (10) included a culture rinsing solution and a cell suspension-diluting medium consisting of 120 mM sodium chloride, 6 mM potassium chloride, 0.1 mM magnesium and calcium ions (MgCl2•6H2O, CaCl2•2H2O), and mixed phosphate (0.6 mM)-phosphite (6 mM) buffer adjusted, respectively, to pH 7.3 and 7.8. These solutions contained 0.1 mM glucose. Salt solution was similar, except that calcium and magnesium ions were 1 mM, and phosphate and phosphite ions 2 and 20 mM respectively. For basal medium the salt solution was supplemented with the vitamins, amino acids, and glutamine of Eagle's medium (3) as purchased from Microbiological Associates (Bethesda, Maryland). Penicillin (100 mg/l), streptomycin sulfate (100 mg/l), and mycostatin (Squibb, 50 mg/l) were added to complete growth medium. In early experiments the energy source in basal medium was provided by 10 mM fructose and 0.1 mM glucose supplemented with 0.1 mM pyruvate and 0.1 mM oxalacetate. This was replaced later by 10 mM glucose without effect on the system. Growth medium consisted of basal medium supplemented with serum as noted.

Serum.—Calf serum was prepared from blood obtained from an abattoir or purchased prepared (Colorado Serum Company, Denver). Human serum prepared aseptically was obtained locally. All serum was filtered through Selas 03 filters, pooled, and dispensed in small amounts and stored frozen at -18° C. until used.

Cells.—HeLa, line 229 of human cervical carcinoma (9), susceptible to polio virus, was obtained from L. McLaren of the Department of Microbiology. Stock cultures of HeLa cells were propagated in basal medium supplemented with 20 per cent calf serum.

Human fibroblast-like cells were obtained from human amnion in primary culture. Membranes from human placenta were obtained immediately...

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after delivery and placed in culture rinsing solution. Membranes were rinsed with culture rinsing solution, the amniotic membrane was stripped from the chorion and rinsed repeatedly with culture rinsing solution to remove blood; then the amniotic membrane was stripped free of clots with forceps and placed in culture rinsing solution containing 0.2 per cent trypsin for 1 hour at 37° C. The membrane was agitated for 3—5 minutes to free cells, and the freed cells were sedimented from the supernatant fluid at 60 × g for 15 minutes. The cell pellet was redispersed in basal medium supplemented with 20 per cent calf serum. Usually epithelial-like cells were not seen after the first or second serial subculture. Fibroblast-like cultures could be maintained by serial subculture from massive inocula through ten or more passages over a 4- to 6-month period. The term “fibroblast-like” cells refers only to the morphologic state of the cells and is not intended to denote a known mesothelial origin.

Cultural procedure.—Stock cultures of HeLa cells and human fibroblast-like cells of amniotic origin were subcultured every 1—3 weeks. Cultures for passage were washed twice with culture rinsing solution, treated for 20 minutes at 37° C. with 0.2 per cent trypsin, pipetted to disperse cells, diluted in growth medium or in cell suspension-diluting medium with 2 per cent fetal calf serum (Hyland Laboratories, Los Angeles), and inoculated in appropriate numbers into warmed Petri dishes containing 5 ml. of medium. Cells were enumerated with a hemocytometer or a Coulter counter, model B. All cultures were incubated at 37° C. ± 0.3° C. in a humidified, continuously changed atmosphere of 2.5 per cent carbon dioxide in air.

Enzymes.—Trypsin (Difco 1:250) was dissolved in culture rinsing solution and stored at -20° C. as a 2 per cent (w/v) solution (10× concentrate). Ribonuclease (Worthington Biochemical Corp., Freehold, New Jersey), 10 mg/ml in 0.9 per cent sodium chloride solution buffered with 0.02 M phosphate at pH 7.2, was stored at 4° C. Deoxyribonuclease (Worthington Biochemical Corporation, type 1) was made up in 1 per cent gelatin in balanced salt solution at pH 7.2. This solution was stored at -70° C. and thawed immediately before use.

Observation of results.—Colonial cultures of fibroblast-like cells were stained with modified Wright’s stain after 10—18 days of incubation when colonies had achieved macroscopic size.

RESULTS

Demonstration of growth-stimulating factor from HeLa cells.—Activity of a growth-stimulating factor for human fibroblast-like cells elaborated by HeLa cells was revealed by the following experiments. Culture dishes were each seeded with 2,000 human fibroblast-like cells from amniotic cultures. Concomitantly, test cultures were seeded with varying numbers of HeLa cells which had received an unfiltered dose of 2,600 roentgens in air delivered from a 220-kv. x-ray machine. In control studies in which a total number of 37 × 10⁶ cells subjected to this dose of radiation were examined, no HeLa cells were found to generate colonies of cells. Although the number of seeded fibroblast-like cells was constant, the number of fibroblast-like colonies with diameters greater than 1 mm. after incubation for 18 days was almost proportional to the number of added irradiated HeLa cells. The dishes in which 20,000; 40,000; and 80,000 radiated HeLa cells were added contained eight, fourteen, and 32 such colonies. The fibroblast-like cells in the control dishes without irradiated HeLa cells usually increased in size during incubation. At the termination of the experiments, the cells were very large in stretched area with relatively small nuclei (Fig. a). In contrast, fibroblast-like cells in colonies in the cultures containing irradiated HeLa cells were much smaller and fusiform in appearance (Fig. b). At termination of the experiments, the majority of the irradiated HeLa cells had degenerated and had become detached from the dish surface. This stimulating effect of radiated HeLa cells on human fibroblast-like cells was observed in eight separate experiments.

Time of release of HeLa factor.—A time study was done to see when HeLa cells elaborated the growth stimulant into cultural fluid. Five ml. of basal medium containing 2 × 10⁶ irradiated HeLa cells was placed in each of a number of culture bottles. Culture fluids were removed and replaced after 2, 4, 6, 9, 12, 18, and 28 days of incubation. Removed fluids were centrifuged to sediment cell debris at 2,000 × g for 10 minutes, and the supernatant fluid was stored at -20° C. Portions of the fluid were diluted equally with fresh growth medium (basal medium with 20 per cent calf serum) for use as growth medium for cultures seeded with 2,000 human amniotic fibroblast-like cells. Control culture of fibroblast-like cells in the basal medium after incubation exhibited only the enlarged cells (Fig. a). Macroscopically visible fibroblast-like
colonies were generated in the presence of 10⁴ irradiated HeLa cells. Although fluid representative of the 4th day of HeLa cell culture produced a few fibroblast-like colonies, appreciable activity of the growth stimulant as judged by colonies over 1 mm. in size appeared first in the fluid representative of the 12th day of HeLa culture (Table 1).

HeLa cultures prepared and treated like those used for elaboration of growth stimulant were analyzed for number of glass-attached cells and their protein content. Protein of cells remaining attached to glass was measured by the Oyama-Eagle modification of the Folin-Ciocalteau reaction (7). Properties of these cultures (Table 1) suggested that elaboration of the growth stimulant occurred during the phase of culture when surviving cells were synthesizing protein, rather than during the early phase when cells were most numerous. Lack of association of factor elaboration with active HeLa

An excerpt from the document: "A portion of the HeLa culture supernate was placed in sterile washed dialyzing membrane in a screw-cap bottle containing an equal volume of fresh growth medium outside the membrane. After 9 days at room temperature, mediums inside and outside the membrane were tested for growth-stimulating activity. The medium outside the membrane contained no activity, whereas the medium inside the membrane promoted colonial growth of the human fibroblast-like cells not seen in the control fibroblast-like cultures in the usual medium—i.e., colonies of fibroblast-like cells over 1 mm. in diameter were 0, 23, and 0 in the control dish, the dish containing the dialyzed medium, and the dish containing the dialysate medium, respectively.

Five-mi. portions of the HeLa culture supernate were adjusted, respectively, to pH 1.75 and 11.8 with 1 N hydrochloric acid and sodium hydroxide, incubated at 37° C. for 60 minutes, and readjusted to pH 7.0. Although the supernate at the usual pH of 7.0 was active, the supernate treated with acid or base was inactive. No colonies were found in the test cultures, but the control culture with untreated HeLa factor contained fourteen macroscopic colonies of fibroblast-like cells. Control growth medium subjected to the same increments of chloride and hydrogen ions supported fibroblast-like growth equally as well as untreated growth medium.

A portion of the HeLa-culture supernate was treated with trypsin (Difco 1:2.50, 0.2 per cent final concentration) for 2 hours at 37° C., and boiled for 15 minutes to inactivate the trypsin. Other portions of HeLa-culture supernate were treated, respectively, with deoxyribonuclease (5 µg/ml final concentration) and ribonuclease..."
**Fig. a.**—Fibroblast-like cell from a control dish. The cell is very large in stretched area with a relatively small nucleus. Note its much greater area than a similar but multiplying cell in Figure b which is at the same magnification. Wright’s stain, x30.

**Fig. b.**—Portion of a colony of fibroblasts from Figure d. Note the typical fibroblast-like appearance. Wright’s stain, x30.

**Figs. c, d, e.**—Effect of trypsin on growth factor. Each dish contained 2,000 human fibroblast-like cells. The control dish (c) contained fresh growth medium to which trypsin was added to a concentration of 0.2 per cent. The dishes of Figures d and e contained growth fluid used to maintain irradiated HeLa cells. This fluid was known to contain the growth factor. To one aliquot of this fluid was added trypsin to a final concentration of 0.2 per cent. Then the control fluid plus trypsin, the growth factor fluid, and the growth factor fluid plus trypsin were incubated at 37° C. for 2 hours. Then all fluids were boiled for 15 minutes to inactivate the trypsin. The fluids were diluted equally with fresh growth medium (basal medium plus 20 per cent calf serum) and used in the experiment. Figure e contains the fresh growth medium plus trypsin. Figures d and e contain the growth factor fluid and the growth factor fluid plus trypsin, respectively. Cultures were incubated 12 days. Colonies were stained with Wright’s stain (shown x6).

**Figs. f, g, h.**—Effect of RNase on growth factor. Each dish contained 2,000 human fibroblast-like cells. To an aliquot of medium used to maintain irradiated HeLa cells and known to contain the growth factor was added ribonuclease (100 μg/ml final concentration). The growth factor aliquot and the aliquot containing the ribonuclease were incubated at 37° C. for 1 hour. The fluids were then diluted equally with fresh growth medium and used in the above experiment. Growth medium was basal medium with 20 per cent calf serum. Figures f, g, and h contain fresh growth medium, growth factor medium, and growth factor medium plus RNase, respectively. Cultures were incubated 16 days. Colonies were stained with Wright’s stain (shown x6).
DISCUSSION

HeLa cells prevented from multiplying by x-radiation have been found to elaborate a factor into culture fluid that stimulated fibroblast-like cells from human amniotic cultures to generate macroscopically visible colonies. Active material was also obtained from lysed HeLa cells, although HeLa cells generating colonies in amniotic fibroblast-like cultures did not produce or give off enough factor to show effect. Since control fibroblast-like cells in the absence of the factor did not degenerate but survived in culture and enlarged and since activity of the factor affected colony frequency more than colony size, it appears that the factor in some manner promoted continuing cell division. The results of preliminary characterization suggested that the factor was not dialyzable, was resistant to boiling and treatment with ribonuclease and deoxyribonuclease, and was susceptible to inactivation by trypsin. These properties would be exhibited by a glycoprotein. Present results suggest that elaboration of the factor in quantity by HeLa cells may not be associated with growth but with degeneration or protein synthesis without cell division. Quantitative biological and immunological assays are needed to further resolve the origin and nature of the factor. Initial observations suggest that factor activity is not limited to the fibroblast-like cells from amniotic cultures; stimulation of colony generation has been observed with single specimens of fibroblast-like cells from human fetal lung and breast cancer tissue.

Growth-stimulating factors for animal cells in culture reported by other workers have included materials nondialyzable as well as dialyzable. A nondialyzable proteinaceous substance described by Alfred and Pumper (1) was elaborated by mouse lung cells adapted to growth in a protein-free medium. This substance, stable at 56°C for 45 minutes, could replace serum in medium for growth of Chang liver cells in culture. Baron and Low (2) reported that skim milk used as a serum substitute for cultural maintenance of cells contained a nondialyzable growth-stimulating substance withstanding boiling for 5 minutes or autoclaving for 15 minutes. An active nondialyzable component of chick embryo extract was described by Kutsky and Harris (6) as a protein extractable together with nucleic acids. By ethanol precipitation of homogenates of human and animal tumors, Kuru, Kosaki, Ito, Matusima, and Matudo (4, 5) isolated a protein substance called "Oncotrophin." Like the substance reported here, this material was nondialyzable, withstood boiling, and was destroyed by acid hydrolysis. Unlike our growth factor, Oncotrophin was not affected by trypsin, although it was destroyed by the bacterial proteolytic enzyme, pronase. The mode of action of these materials and of our stimulating factor is unknown.

REFERENCES

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