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A Diffusible Factor Restoring Contact Inhibition of Growth to Malignant Melanocytes

(conditioned medium/orientation/saturation density)

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ABSTRACT **Contact inhibition of growth is the property *in vitro* whose loss is most closely correlated with tumorigenicity *in vivo*. A contact-inhibited melanocytic cell line produces a diffusible protein-containing factor capable of restoring contact inhibition of cell division to highly malignant hamster melanocytes. Its addition to subconfluent cultures of the malignant cells is followed by the obligatory acquisition, at confluence, of the contact-inhibited state. Cultures are flat, oriented, and fibroblast-like, and show a 55% decrease in saturation density with no loss of viability. The effect is reversible. Present in conditioned media of cultures of the contact-inhibited melanocytic cell line, isolated by column chromatography on Sephadex G-200, the factor appears to be of high molecular weight. Activity is preserved in aqueous solutions at 4° for at least 8 weeks, but is destroyed by repeated freeze-thawing or by treatment with Pronase.**

This newly recognized contact-inhibitory factor may be a prototype for a more general and fundamental mechanism for regulation of normal cell–cell interactions. It is the first cell-elaborated factor to be isolated that is capable of restoring the capacity for contact inhibition of growth to malignant cells.

One of the characteristic properties of benign cells *in vitro* is that of contact inhibition of growth (1) (density-dependent inhibition of growth) (2). Cells reaching confluence cease proliferating in response to unknown signals generated by the interaction of direct contact factors (3–5) and modifying constituents of the culture medium, especially serum (6–8). Virally-transformed cells appear incapable of generating and/or responding to such signals and fail to cease growing upon reaching confluence, but form, instead, multilayered cultures with characteristically higher saturation densities than in the nontransformed cultures. Contact inhibition of growth is the property *in vitro* whose loss is most closely correlated with tumorigenicity (9), and among malignant cell lines there is a direct relationship between saturation densities of cultures and efficiency of transplantability *in vivo* (10).

In view of the mounting evidence that malignancy involves aberrations either of genetic information or of epigenetic factors controlling its expression, it would not be surprising to find macromolecules present in benign cells specifically concerned with the maintenance of the capacity to undergo contact inhibition of growth. Introduction of such macromolecules into malignant cells might restore to such cells the differentiated properties that they lack. We report here the identification and isolation, from cultures of a contact-inhibited melanocytic cell line, of a protein-containing macromolecule that is capable of reversibly inducing in highly malignant melanocytes the capacity for contact inhibition of growth.

The origin of a contact-inhibited, aneuploid, hamster amelanotic melanoma cell line has been described (11). It arose during experimental pigment transformation of a highly malignant, hamster amelanotic malignant melanoma cell line (RPMI no. 1846) by nucleic acids derived from 7,12-dimethylbenz(a)anthracene-induced benign blue nevi of hamsters. In addition to pigmented transformants with markedly (80%) reduced proliferative rate, amelanotic melanocytes (cell line FF) appeared which exhibited *in vitro* and *in vivo* changes that have persisted as stable, hereditary characteristics for almost 4 years. Whether the amelanotic cells were also true transformants or simply the consequence of a selection process occurring after treatment of the original cell line (RPMI no. 1846) with nucleic acids from blue nevi is not certain, but this question is not crucial to our observations. *In vitro*, cultures were contact-inhibited, flat, very cohesive, and much more resistant to proteolytic dispersion than their malignant precursors. Cytophotometric examination showed the FF cells to have a hypertetraploid complement of DNA, in contrast to the hyperoctaploid content of the parental cells (12). *In vivo* transplantation of FF cells gave a significant delay in appearance of malignant tumors, with prolonged survival of recipients (13). Cytophotometric evaluation of cells *in vitro* from the "delayed" tumors revealed subtetraploid DNA (12), so that delayed tumors appeared to have arisen after loss of DNA from some of the injected cells.

Three types of evidence suggested that the contact-inhibited (FF) cell cultures might be producing a diffusible inhibitor substance capable of influencing growth and morphology of RPMI no. 1846 cells. First, morphologic and cytometric studies showed the presence in the contact-inhibited cultures of occasional larger, bizarre-looking cells with morphology and DNA content resembling that of the parental RPMI no. 1846 line (12). Yet such cells failed to overgrow the contact-inhibited FF cultures. Second, a 50:50 mixture of regular medium and 48-hr-old medium from contact-inhibited (FF) cultures ("conditioned" medium) produced a 45% reduction in saturation densities of freshly

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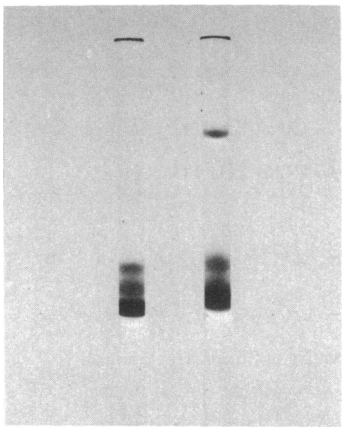

FIG. 1. Polyacrylamide gels showing electrophoretic separation of proteins present in serum-free conditioned medium from cultures of RMPI no. 1846 cells (*left*) and FF cells (*right*). Electrophoresis at 10 mA per tube for 4 hr. Gels stained for 40 min with 0.25% Coomassie brilliant blue in H_2O–methanol–acetic acid 5:5:1, destained in H_2O–methanol–acetic acid 17:1:2 overnight.

plated cultures of RPMI no. 1846 cells, whereas similarly conditioned medium from RPMI no. 1846 cultures had no effect upon growth of FF cells. Third, FF-conditioned medium produced a 2.5-fold increase in the percentage of fibroblast-like forms in cultures of RPMI no. 1846 cells. An attempt was made, therefore, to examine conditioned media from both cell lines for qualitative differences in proteins.

MATERIALS AND METHODS

Preparation of the Starting Material. Saturated cultures both of the amelanotic parental cells (RPMI no. 1846) and of the blue nevus-transformed (FF) cells were grown for 48 hr in the usual growth medium (RPMI no. 1640), but without serum. After removal of the rare detached cells by centrifugation at 1500 rpm, the supernatants were lyophilized in batches of 60 ml. The residue was dissolved in an appropriate buffer and used for polyacrylamide gel electrophoresis and column chromatography, or was first dissolved and dialyzed in 1 mM phosphate buffer, pH 7.1, for 4 hr to remove substances of small molecular weight.

Polyacrylamide Gel Electrophoresis. For the analytical polyacrylamide gel electrophoresis, gels were prepared as described (14). The concentrated material was made up with 1% sodium dodecyl sulfate, 1 mM 2-mercaptoethanol, and 6% sucrose; 40–60 μg per 40–60 μl were layered on the gels, which were 9 cm in length and 0.4 cm in diameter. The amount of the biologic material was estimated by the absorbance at 280 nm with albumin as a standard. The final gel composition was 5% acrylamide (recrystallized), 0.12% *N,N'*-bis-methylene acrylamide, 0.1% Na dodecyl sulfate, 0.1 M sodium phosphate buffer, pH 7.1, 0.05% *N,N,N',N'*-tetramethylenediamine, and 0.075% ammonium persulfate. The electrode buffer was 0.1 M sodium phosphate, pH 7.1, containing 0.1% Na dodecyl sulfate. Electrophoresis was carried out at 10 mA per tube for 4 hr. The gels were stained for 40 min in 0.25% Coomassie brilliant blue in water–methanol–acetic acid 5:5:1, and destained in water–methanol–acetic acid 17:1:2 overnight.

Fractionation on Sephadex G-200. Sephadex G-200 in 0.1 M Tris·HCl buffer, pH 8.0, containing 1 M NaCl was poured into 2 × 100-cm columns and equilibrated with the same buffer. Lyophilized supernatants were dissolved in 5 ml of buffer, dialyzed against 0.15 M NaCl overnight, clarified by centrifugation at 17,000 rpm for 10 min, concentrated, and applied to the column in a cold room. Fractions of 1 ml were collected, and absorbance was measured at 280 nm. The fractions of particular peaks were pooled, concentrated, and dialyzed against phosphate buffer 0.1 M, pH 7.1.

Examination of Isolated Fractions for Other Substances. The isolated fractions of peaks I and II obtained by Sephadex chromatography (see *Results*, below) were examined for protein by the method of Lowry *et al.* (15), for RNA by the orcinol method (16), and for DNA by the diphenylamine method (17). The 80,000 g pellet both from pooled, FF-conditioned medium and from freeze-thaw-ruptured FF cells, as well as concentrated "upper band" material eluted directly from polyacrylamide gels, were examined for virus particles by negative staining in a Phillips 201 electron microscope.

Tissue Culture Trials. Freshly prepared material from Peaks I and II was sterilized by precipitation in ethanol, redissolved in sterile, calcium- and magnesium-free phosphate-buffered saline (Gibco), pH 7.2, at a concentration of 0.5 mg/ml, and diluted 1:10 in the complete growth medium (RPMI no. 1640, 10% fetal-calf serum, and antibiotics) just before use. Freshly trypsinized suspensions of RPMI no. 1846 cells were plated out in multiple T30 Falcon flasks with initial inocula of 250,000 cells per flask with or without addition to the media of the peak-I material to be tested at a final concentration of 50 μg/ml. Other cultures contained peak-I material that had been treated with either Pronase (30 μg/ml at 37° for 8 hr) or DNase (10 μg/ml at 37° for 15 min, with added 5 mM $MgCl_2$). In addition some cultures received 50 μg/ml of the combined proteins from the second main peak (peak-II material) eluted from Sephadex G-200 columns. (This appeared as the "lower bands" on analytic polyacrylamide gels.) The cultures were fed with the growth medium and fractions twice weekly; maximum growth was reached in 5–7 days.

RESULTS AND DISCUSSION

Fig. 1 shows the polyacrylamide gel electrophoretic separation of the proteins present in the serum-free conditioned media

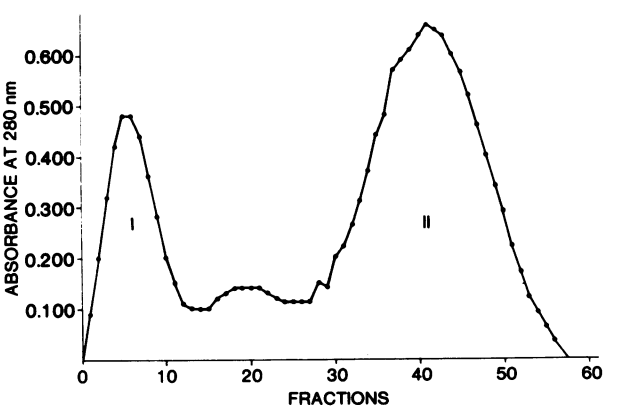

FIG. 2. Separation of fractions on Sephadex G-200 of lyophilized supernatant from the contact-inhibited FF cell cultures. Absorbance was measured at 280 nm for each 1-ml fraction.

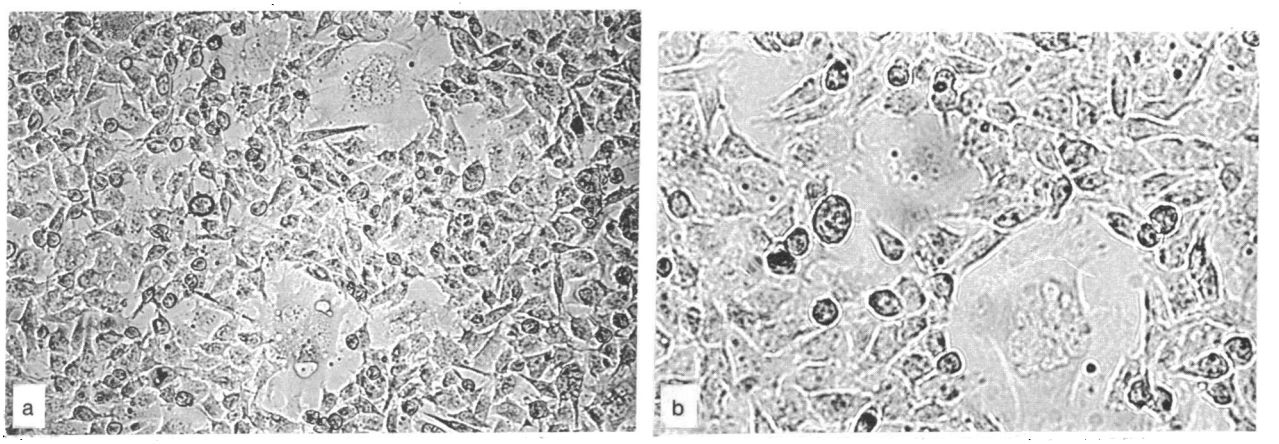

FIG. 3. RPMI no. 1846 culture, 10 days old, confluent. No contact-inhibitory factor added to medium. (*a*) ×52; (*b*) ×276.

from RPMI no. 1846 and FF cell cultures. The upper band of the FF gels is not seen in the RPMI no. 1846 gels. Fig. 2 shows the separation on Sephadex G-200. Three separate experiments showed that the observed effects upon contact inhibition of growth (see below) were associated only with the first peak (I). The second peak (II) contained the lower bands visible in both RPMI no. 1846 and FF gels and was inactive with respect to contact inhibition. Reactions were positive for protein in both, and reactions with orcinol were negative. Both peaks gave diphenylamine reactions, indicating the presence of deoxyribose, but electron microscopic search of all negative-stained preparations failed to reveal any virus particles. Results of tissue culture trials are summarized in Table 1.

Cultures of RPMI no. 1846 cells containing added material from peak I ("factor") failed to exhibit, at confluence, the usual appearance of disorientation and multilayered piling up of rounded or dendritic melanocytes (Fig. 3), but instead consisted of well-oriented, fibroblast-like cells growing in monolayers (Fig. 4) and exhibiting contact inhibition of growth similar to that found in cultures of FF cells (Fig. 5). Measurement of saturation densities showed an average 55% decrease in the contact-inhibited cultures compared to controls without peak-I material. The former did not exceed 1.6 × 10^6 cells per flask (with 4 ml of medium), while the latter averaged 3.6 × 10^6 cells per flask. Appearance of increased numbers of fibroblast-like cells in part preceded actual con-

TABLE 1. *Induction of contact inhibition of growth in RPMI no. 1846 cultures*

Fraction of FF-conditioned medium added	Treatment	No. of cultures	No. showing contact inhibition of growth	Saturation densities (×10^6)* (average)
None	—	10	0	3.6
Peak I	None	14	14	1.6
	DNase	5	5	—
	Pronase	5	0	—
	4° for 8 weeks	4	4	—
	Freeze-thaw 3 times	4	0	—
Peak II	None	10	0	4.3

Freshly trypsinized RPMI no. 1846 cells were plated out in Falcon T30 plastic flasks at initial densities of 250,000 cells per flask in 4 ml of growth medium (RPMI no. 1640, 10% fetal-calf serum, antibiotics). Cultures were incubated at 37° in a humidified atmosphere and with added 5% CO_2 in balanced air. Media were changed twice weekly until confluence was reached. Duplicate cell counts were obtained in a hemocytometer chamber on combined supernatants and attached cells for each flask.

* Values should be multiplied by 10^6.

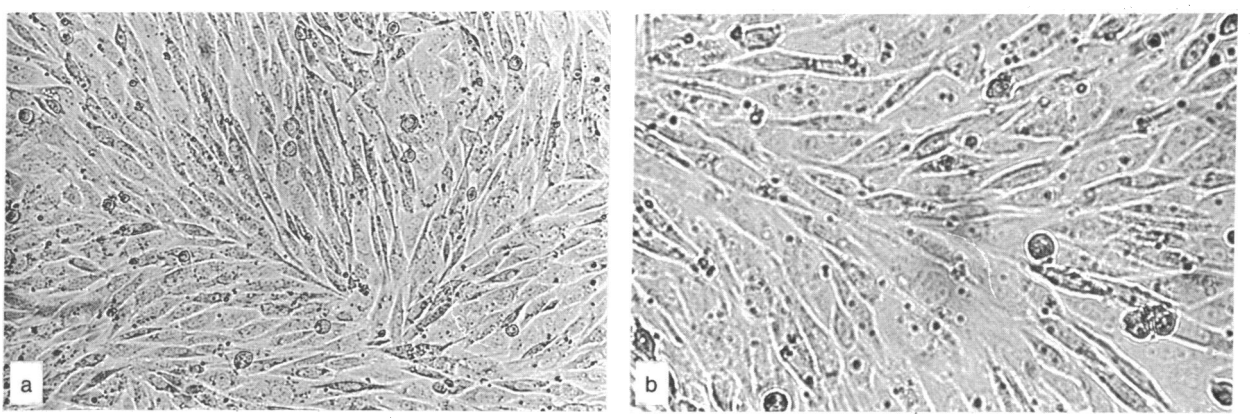

FIG. 4. RPMI no. 1846 culture, 10 days old, confluent. Grown in medium containing added contact-inhibitory factor, 50 μg/ml. (*a*) ×52; (*b*) ×276.

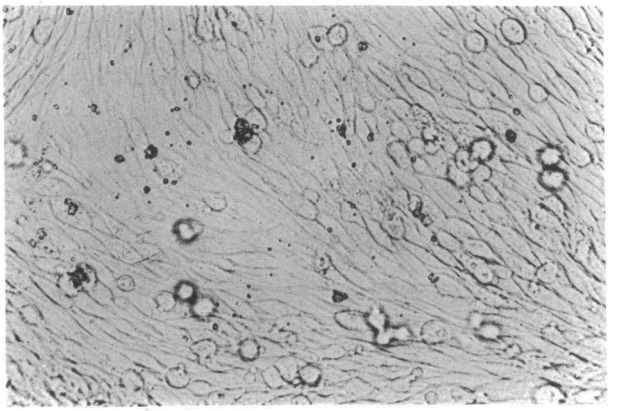

FIG. 5. FF culture, confluent. ×110.

tact between cells, but the number greatly increased following such contact. Prior treatment of peak-I material with DNase had no effect upon contact inhibition, but prior treatment with Pronase completely prevented it. Material from peak II not only failed to promote contact inhibition but even caused some stimulation of growth.

The changes described were completely reversible. Replacement of "factor"-containing medium with "factor"-free medium was followed, within less than 24 hr, by reversion of cells to the dendritic shape, and by the appearance of the disoriented, multilayered morphology, as cells resumed further growth. However, such disoriented cultures again responded to the later readdition of "factor," although this required, in heavily overgrown cultures, up to a week or more. Cell viability in contact-inhibited cultures, as judged by exclusion of Trypan Blue, was the same as in controls, 91%. The biologic effectiveness of the "factor" was preserved in complete culture medium at 4° for at least 8 weeks, the longest period tested, but was destroyed by as few as three repeated freeze-thawings.

Although the contact-inhibitory fraction obtained by Sephadex chromatography had, in association with the protein, some deoxyribose-containing material, the possibility that DNA may be associated with the contact-inhibitory activity is unlikely in view of the failure of prior treatment with DNase to destroy the biologic effect. Deoxyribose-containing material was also present in the second peak fraction from Sephadex chromatography, but the latter fraction had no contact-inhibitory activity.

It is not known whether this newly identified "contact-inhibitory factor" is detectable either intra- or extracellularly in *diploid* melanocytes, such as blue nevus cells, or in other biologically benign cell types. It may be present in diploid cells only intracellularly, having become "unmasked" in diffusible extracellular form only by virtue of "leakiness" of the aneuploid transformants. "Leakiness" of malignant cells in comparison to nonmalignant cells has been reported with respect to several cytoplasmic enzymes (18). It has also been shown that the growth of polyoma-transformed BHK 21 cells is rapidly inhibited in mixed cultures with normal static mouse fibroblasts, but no evidence could be found for the presence of a stable, diffusible inhibitor released into the medium (19). It was considered possible that such hypothetical inhibitors might be transferred directly from cell to cell in a manner analogous to that already shown to occur in situations of "metabolic cooperation" (20, 21).

Nor is the specificity of contact-inhibitory factor known with respect to other malignant cell types. It clearly differs from the diffusible extracellular factor reported from another aneuploid, contact-inhibited cell line, 3T3 (22), in that the latter factor sustains contact inhibition of growth in the nonmalignant 3T3 cells but cannot impose it upon viral-transformed malignant 3T3 cells or upon the malignant epithelial HeLa cell line.

The existence of a readily reversible mechanism controlling regulation of normal cell–cell interactions may be inferred from the ability of contact-inhibited cells to undergo rapid escape from the stationary to the proliferative state after either brief proteolytic treatment (23) or addition of higher concentrations of fresh serum (5, 6). The reverse is also true, since morphologic changes associated with contact inhibition of growth may develop rapidly after such stimuli as addition to the environmental milieu of cyclic AMP (24, 25). On the basis of current evidence, it seems most likely that any such regulatory mechanism is expressed through rapid alterations in the cell surface. It is possible that contact-inhibitory factor acts in melanocytes to prepare the cell surface to respond appropriately to cell contacts; malignant melanocytes, lacking contact-inhibitory factor, fail to modify their surfaces appropriately to receive signals generated by the interaction of cell contacts and environmental modifiers. In recent preliminary experiments, not yet published, evidence has been found that the cell surfaces of the malignant (RPMI no. 1846) and contact-inhibited (FF) melanocytic cell lines do indeed differ from each other with respect to the presence of a specific protease, cathepsin B1, and further preliminary studies suggest that one effect of contact-inhibitory factor upon malignant cells is to modify availability of this protease at the surface (26). We suggest that contact-inhibitory factor may be the prototype for a fundamental mammalian cellular mechanism of general biologic significance, for the regulation of normal cell-to-cell contact interactions. Although contact inhibition of growth may be induced in malignant cells by other exogenous substances, this is the first demonstration of such activity by a discrete protein-containing factor produced by contact-inhibited cells. Contact-inhibitory factor may provide a useful new tool for the further elucidation of the complex sequence of events involved in the establishment of the contact-inhibited state. It also provides, for the first time, a factor potentially capable of restoring to malignant melanocytes a critical normal cell function *in vitro* whose absence is closely related to the malignant state *in vivo*. The identification and isolation of such growth-regulating macromolecules present formidable challenges to the cancer biologist, but the present findings at least lend some encouragement to such efforts and to the belief that functional repair of malignant cells may ultimately be achievable.

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