Differential Control by Platelet Factors of Squamous Differentiation in Normal and Malignant Human Bronchial Epithelial Cells

John F. Lechner,1 Irene A. McClendon, Moira A. LaVeck, Abulkalam M. Shamsuddin,2 and Curtis C. Harris

Laboratory of Human Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20205

ABSTRACT

Recently, we developed a nutritionally optimal medium for rapid clonal growth (>1 population doubling/day) of normal human bronchial epithelial (NHBE) cells. Adding fetal bovine or adult human blood-derived serum to this medium depresses the clonal growth rate of NHBE cells in a dose-dependent fashion. In contrast, 10 representative lines of human lung carcinomas either replicate poorly or fail to grow at all when inoculated at clonal density in serum-free medium, and their rates of multiplication increase in direct proportion to the amount of blood-divided serum added to the optimized medium. Thus, the growth factor requirements of these lung carcinoma cell lines are significantly different from those of their normal counterparts. Blood-divided serum reduces the clonal growth rate of NHBE cells by specifically inducing the normal cells, but not lung carcinoma cells, to undergo squamous differentiation. The differentiation-inducing activity was found in platelet lysates. In addition, a growth-inhibiting activity that did not induce squamous differentiation of NHBE cells was also identified in partially purified commercial preparations of platelet-derived growth factor. This observation was in marked contrast to results using human bronchial fibroblasts and human lung carcinoma cell lines; the growth rate of the former was significantly stimulated by commercial preparations of platelet-derived growth factor, whereas the growth rates of the tumor cell lines were unaffected. These results indicate that an aberration in the cellular differentiation as assayed in vitro is positively correlated with cancer and suggests that decreased responsiveness to inducer(s) of differentiation may be a major aspect of bronchial cell carcinogenesis.

INTRODUCTION

Numerous investigations delineating differences that demark cancerous from normal cells have most commonly noted a reduced dependency for peptide mitogens (3, 4, 10, 14, 16, 17), although changes in nutritional (10) and ion (9, 10) requirements for multiplication have also been documented. Results of this nature have provided the basis for an often-suggested hypothesis to explain carcinogenesis, i.e., the significant difference between normal and tumor cells is a reduced requirement for exogenous mitogens and nutrients in order for the latter to initiate multiplication. This reduced-growth-requirements hypothesis agrees with the data obtained with most fibroblastic cell model systems. However, the majority of human cancers are carcinomas, and a basic difference between many fibroblasts and epithelial cells is that the latter often terminally differentiate. Thus, fibroblastic cell models cannot adequately evaluate the role of differentiation in growth control. Recently, cell culture models having the capacity to terminally differentiate have been developed, and some investigators noted that an aberration in the differentiation program is often correlated with transformation (5, 12, 13, 18, 20–22, 25, 26).

Recent advances in culture techniques for NHBE3 cells have resulted in a nutritionally optimized, serum-free medium for replicative cultures of these cells (7). We have now focused on medium additives which affect the degree of NHBE cell squamous differentiation. This report shows that a platelet factor(s) can inhibit multiplication and induce squamous differentiation of NHBE cells, and documents that cells from human lung carcinomas are not similarly affected by platelet extracts. The results support the hypothesis that aberrant differentiation control is a major step towards malignant transformation of bronchial epithelium.

MATERIALS AND METHODS

Cells and Media. Methods used to establish replicative cultures of both NHBE and fibroblastic cells have been published previously (6, 7). The cells were grown in surface-coated (fibronectin, 10 μg/ml; bovine serum albumin, 10 μg/ml; Vitrogen collagen, 30 μg/ml) culture dishes (7) containing serum-free LHC-4 medium. This medium was prepared by reducing the osmolality of LHC-1 medium (7) from 345 to 310 mOsmol/kg (by adding 0.1 volume of distilled H2O), and adding 1 × 10−4 M 3,3′,5′-triiodothyronine and aqueous extracted bovine pituitary protein (35 μg/ml) (7). The bronchial fibroblasts and lung carcinoma cultures were routinely grown in MCD 105 medium (11) with 2.5% fetal bovine serum and Roswell Park Memorial Institute Medium 1640 with 10% fetal bovine serum, respectively.

The lung carcinoma cell lines used and their sources were: A549 (American Type Culture Collection, Rockville, Md.); Calu-1, Calu-6, SK-MES-1, and SW 900 (J. Fogh, Sloan-Kettering Institute for Cancer Research, Rye, N. Y.); Hut 292, NCI-H23, and NCI-H157 (A. Gazdar, National Cancer Institute-Navy Medical Oncology Branch, Bethesda, Md.); and A2182, A427, A1188, and A1146 (S. Aaronson, National Cancer Institute, Bethesda, Md.).

Epidermal growth factor and CPDFG preparations were purchased from Collaborative Research, Waltham, Mass., and Bethesda Research Laboratories, Inc., Gaithersburg, Md.; bovine insulin was a gift of Eli Lilly Co., Indianapolis, Ind.; HPPDGF was a gift of Dr. N. H. Antoniades, Harvard University School of Public Health, Boston, Mass.; clinically outdated platelets were obtained from the NIH Clinical Center, Bethesda, Md.; the other medium supplements were obtained from Sigma Chemical Co., St. Louis, Mo. Nutrient media and fetal bovine serum were purchased from Biofluids, Inc., Rockville, Md.

BDS was obtained from coagulated fetal bovine or human blood.

1 To whom requests for reprints should be addressed at NIH, National Cancer Institute, Laboratory of Human Carcinogenesis, Building 37, Room 216, Bethesda, Md. 20205.
2 Present address: Department of Pathology, University of Maryland School of Medicine, Baltimore, Md. 21201.
3 The abbreviations used are: NHBE, normal human bronchial epithelial; CPDFG, partially purified commercial platelet-derived growth factor; HPPDGF, highly purified platelet-derived growth factor; BDS, blood-derived serum; PDS, plasma-derived serum; dThd, deoxthymidine.

Received April 4, 1983; accepted August 26, 1983.
Subsequently, BDS was passed over a Sephadex G-10 column to remove small molecules and ions (4, 6, 7). PDS was prepared from anticoagulated (heparin) blood. The formed cellular elements were removed by centrifugation (15000 × g for 7 min) before coagulation by dialysis against 250,000 volumes of H2O. The serum was then passed over a Sephadex G-10 column to remove Ca2+. Sera were stored as 50-mL aliquots at −70°C until used and were thawed only once. Human platelets were lysed by freeze-thawing 3 times. The preparation was then centrifuged (50,000 × g for 30 min), dialyzed against 200,000 volumes of H2O, and filtered through a 0.22-µm filter before use. This crude preparation contained 6.7 mg protein per ml.

**Growth Measurements.** The mitogenic potency of medium supplements was measured using a clonal growth rate assay described previously (4–8). Five thousand NHBE (colony-forming efficiency, 2 to 5%) or 1000 carcinoma (colony-forming efficiency, 10 to 25%) cells were inoculated per 60-mm dish. After 8 to 11 days of incubation, the colonies were fixed with 10% formalin and stained with 0.25% aqueous crystal violet. The mean number of cells per clone in 18 randomly selected colonies (5/replicate dish) was determined for each additive concentration. To derive the growth rate (mean cell generations/day), the log of the average number of cells per clone was divided by the number of days of incubation. Student's t test was used to evaluate the significance of difference between experimental groups (2).

In order to determine the effect of medium additives on the rate of nucleic acid synthesis, 20,000 cells were inoculated per coated 35-mm culture dish containing serum-free LHC-4 medium supplemented with [14C]dThd (10 nCi/ml; 52 mCi/mmol). After 2 days of growth, the cultures were rinsed twice with nonradioactive medium and inoculated for 1 day in isotope-free medium with the indicated amount of supplement. For DNA synthesis, the cultures were then incubated for 2 additional days in media containing the same concentration of supplement and [3H]dThd (2 µCi/ml; 75 Ci/mmol). The cultures were then rinsed twice with Dulbecco's phosphate-buffered saline and dissolved in 1 ml of 0.2 N NaOH containing calf thymus DNA (40 µg/ml). A mixture of NaOH and tissue was poured onto a 1 N HCl-soaked Whatman GF/C glass filter which was rinsed with 100% ethanol, and the radioactivity was assayed in a scintillation counter. The [3H]/[14C] dpm ratio was used as a measure of the amount of DNA synthesized, and this value was compared to the ratio obtained for the control cultures. RNA synthesis was measured using the same protocol except: (a) [3H]uridine (6 µCi/ml; 25.5 Ci/mmol) was substituted for [3H]dThd; (b) [3H]uridine was added as a 4-hr pulse 3 days after changing the cultures to supplement containing media; (c) and the cells were lysed using a solution of 2% sodium dodecyl sulfate, bovine serum albumin (0.1 µg/ml), and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline (7), pH 7.6.

The same protocols were used for control dishes except that a pair of cultures was harvested after 1 day of incubation in isotope-free medium, and other pairs of cultures that were not incubated with the [3H]-labeled nucleotides were harvested concurrently with the experimental cultures. These 2 sets of controls served to measure the stability of the [14C]-labeled DNA/culture for the duration of the experiment; no significant differences ([14C] dpm) between early and late controls were noted.

**Morphological Characterization.** Cell morphology was monitored by phase-contrast microscopy. Cultures were also examined using transmission electron microscopy and scanning electron microscopy (6). An image analyzer (Artrek 800) was programmed to count the number of cells per colony and to measure cell area and distance between adjacent cell perimeters within a colony. Eighteen randomly selected, fixed, and stained colonies were analyzed per experimental variable. The percentage of cells capable of making crossing envelopes was ascertained using a modification of the method of Sun and Green (23).

**RESULTS**

The Differential Effect of BDS on NHBE and Lung Carcinoma Cells. The effect of serum on the clonal growth rate of NHBE cells was ascertained by dose-response experiments. Adding as little as 2.5% of either bovine or human BDS to LHC-4 medium significantly reduced the mean clonal growth rate; this inhibition increased in proportion to the BDS concentration (Chart 1). DNA synthesis measurements corroborated the data shown in Chart 1; the amount of [3H]dThd incorporated into acid-precipitable DNA was reduced to less than 15% as a result of adding BDS to the cultures, but RNA synthesis was not markedly affected (Table 1).

Cells maintained for 8 to 10 days in serum-free medium were migratory, exhibited an ovoid morphology, and had few tonofilaments and desmosomes (Fig. 1, a and c; Table 1). On the other hand, large, flat, closely adjacent cells with numerous junctional complexes and extensive networks of intermediate filament bundles were observed in cultures maintained in BDS-containing medium (Fig. 1, b and d; Table 1). In addition, 80% of the cells maintained for 8 to 10 days in BDS-containing medium could be induced by exposure to ionophore A23187 (23) to

![Chart 1. Mitogenic potency of human BDS and PDS was measured using a clonal growth dose-response assay (8). Five thousand cells were inoculated per dish. The colonies were fixed and stained after 8 to 11 days of incubation. The growth rate is defined as the number of population doublings per day. To measure this, the average log of the number of cells per 18 randomly selected colonies was determined and divided by the number of days of incubation. The line describing the data was derived using variance weighted least-square regression equations (2). Student's t test was used to evaluate significance of difference between experimental groups. Results using bovine BDS and PDS were statistically identical to those using human BDS and PDS.](chart1.png)

**Table 1. Effect of culture conditions on nucleic acid synthesis, cell surface area, and cell migratory activity of NHBE cells**

<table>
<thead>
<tr>
<th>Additive</th>
<th>DNA synthesis (%)</th>
<th>RNA synthesis (%)</th>
<th>Cell surface area (sq µm)</th>
<th>Intercellular distance (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>1250</td>
<td>23</td>
</tr>
<tr>
<td>8% BDS</td>
<td>83</td>
<td>ND</td>
<td>1400</td>
<td>6</td>
</tr>
<tr>
<td>8% BDS</td>
<td>83</td>
<td>ND</td>
<td>1400</td>
<td>6</td>
</tr>
<tr>
<td>PDS</td>
<td>89</td>
<td>ND</td>
<td>1400</td>
<td>6</td>
</tr>
<tr>
<td>CPDGF (3 units/ml)</td>
<td>6</td>
<td>107</td>
<td>5500</td>
<td>70</td>
</tr>
</tbody>
</table>

*Mean distance between adjacent cell perimeters within individual colonies.*

**Results with additive were significantly different (p < 0.05) from results without additive.**

**ND, Not done.**
develop cross-linked envelopes; less than 5% of control cells that were incubated in serum-free medium (minus ionophore) formed envelopes.

Human lung carcinoma lines were also incubated in LHC-4 medium without and with 8% BDS. The results (Table 2) documented that serum toxicity per se was not responsible for the observed inhibition of NHBE cell growth; all lines divided significantly (p < 0.05) more rapidly in BDS-supplemented medium except for line A 427 (which failed to grow at clonal density in LHC nutrient media).

**Differential Effect of Platelet Factors.** In order to delineate the constituent(s) of BDS causing NHBE cells to undergo squamous differentiation, various blood fractions were incorporated into LHC-4 medium. The results with BDS were markedly different. Whereas BDS was progressively growth-inhibitory, PDS (prepared from either bovine or human blood) had no significant effect on the clonal growth rate (Chart 1). PDS did, however, decrease migratory activity (Table 1) and affect the cell-cell arrangement within colonies; i.e., cells were commonly in apposition, but junctional complexes were rarely found.

Since BDS did not depress the clonal growth rate, platelet constituents were tested. Medium supplemented with crude platelet extract (>0.3%; 20 µg protein/ml) completely abolished clonal growth and induced the cells to undergo squamous differentiation. (Heparin was also tested and was found to have no significant effect on the clonal growth rate of NHBE cells.) Thus, commercial (partially purified) preparations of platelet-derived platelet factor were tested. Cultures were incubated for 5 days in serum-free LHC-4 medium and were then exposed to serum-free LHC-4 containing CPDGF (3 units/ml; 1 unit in culture medium with 0.4% bovine calf serum stimulates [3H]dThd incorporation by confluent BALB/c 3T3 cells equal to that in medium containing 10% fetal bovine serum). These cells became highly migratory, and their cross-sectional area increased more than 4-fold (Table 1). After 2 days of incubation, the cells became quiescent, and DNA synthesis ceased (Table 1). However, the differentiation process was incomplete. The cells acquired only some of the other characteristics exhibited by cells incubated in BDS-containing medium; i.e., the cells were neither flat nor multilayered (Fig. 2e), and less than 10% of the cells were capable of forming cross-linked envelopes after 8 days of incubation in CPDGF-containing medium. Perinuclear vacuoles accumulated in cells incubated in either BDS or CPDGF-containing medium. However, instead of extensive networks of intermediate filament bundles and numerous desmosomes, CPDGF-exposed cells (Fig. 2b) had numerous microvilli, few desmosomes, and a readily noticeable band of short tonofilament bundles circumcribing the perinuclear vacuoles. The cytoplasm beyond this filament band was virtually occupied by numerous free ribosomes and polyribosomes; but only a few mitochondria were found in this zone. Nuclear changes were also noted; the nucleus was often lobular, and nucleoli were rarely seen.

The effect of CPDGF (3 units/ml) on NHBE and fibroblastic cell DNA synthesis was compared, and dissimilar results were obtained (Chart 2). Whereas the expected stimulation of DNA synthesis in the fibroblasts occurred, the preparation inhibited epithelial cell DNA synthesis in a dose-response manner. The effect of CPDGF on several human lung carcinoma cell lines was also ascertained. The rate of DNA synthesis of all of these cell lines was unaffected by a 2-day exposure to CPDGF (3 units/ml) (Table 3). Statistically identical results (data not shown) were obtained with different lots of CPDGF preparations purchased either from Collaborative Research or Bethesda Research Laboratories.

The kinetics of inhibition of DNA synthesis were ascertained by measuring the amount of [3H]dThd incorporated into DNA per 3 H/14 C ratio. The methods were as described in "Materials and Methods" and the legends of Tables 1 and 3.
DISCUSSION

We have established nutritionally optimized serum-free culture conditions for replicative cultures of NHBE cells (7). Factor(s) in BDS rapidly cause these cells to cease DNA synthesis and cytokinesis and acquire properties associated with squamous differentiation of bronchial epithelium (24). These cells become flat, contain an extensive network of intermediate filament bundles, have numerous desmosomes, and tend to be multilayered, and a high percentage of cells are capable of synthesizing cross-linked envelopes. The response of human lung carcinoma cells was markedly different. Ten representative types grew moderately rapidly at clonal densities when inoculated in this medium with BDS, but replicated either poorly or failed to grow at all in serum-free medium. Thus, the carcinoma cells appear to have gained the capacity to respond to mitogens known to be present in BDS. This observation is in contrast to the results obtained with many other cell systems and emphasizes the point that the growth factor requirements of the carcinoma cells may not necessarily be less complex than those of their normal counterparts.

Altered factor and nutrient growth requirements for malignant cells are a well-documented phenomenon and have provided an often-expounded hypothesis to explain carcinogenesis, i.e., that the significant difference between normal and tumor cells is a reduction in nutrient and/or BDS growth factor requirements by the tumor cells in order to proliferate (3). This hypothesis remains a plausible mechanism for describing the malignant transformation of fibroblastic cells. However, our results comparing normal and malignant human bronchial epithelial cells supports an alternative hypothesis; e.g., the tumor cells have lost the ability to respond to the differentiation-inducing factor(s) which control growth of the normal cells.

Recently, several investigators have focused on the control of differentiation in normal epithelial cells and have shown that an aberration in the differentiation program as assayed in vitro is positively correlated with malignant transformation. Stanbridge et al. (12, 21) reported that nontumorigenic HeLa-keratinocyte hybrids xenotransplanted into nude, athymic mice divided a few times and then terminally differentiated. In contrast, tumorgenic hybrids evaded the inducements to differentiate. In vitro assays have also been described. Kulesz-Martin et al. (5) and Yuspa and Morgan (26) noted that normal murine epidermal keratinocytes cease proliferating and undergo squamous differentiation when the Ca\(^{2+}\) concentration of the medium is increased above 0.1 mmol. Pretreatment of epidermal tissue or treatment of keratinocyte cultures with carcinogens yielded cultures exhibiting a significant increase in the number of colonies which were resistant to induction of squamous differentiation by high concentrations of Ca\(^{2+}\). Scott et al. (18) and Wille et al. (25) have described a correlation between neoplastic transformation and defective differentiation of murine BALB/c 3T3 T-proadipocytes. Human plasma contains factors which induce the nontransformed cells to growth arrest and then terminally differentiate. In contrast, spontaneous, chemical, or viral transformed proadipocytes neither undergo growth arrest nor do they terminally differentiate in the presence of either barium-adsorbed or complete human plasma.

Our results are compatible with the suggestion that aberrant differentiation control is positively correlated with carcinogenesis; i.e., the malignant lung cells have a reduced ability to respond to
the factors that induce NHBE cells to undergo terminal differentiation. However, the nature of the differentiation-inducing signals for the NHBE cells is different than described for the other systems. Previously, we showed that raising the Ca\(^{2+}\) concentration above 100 µM did not depress the growth rate or induce squamous differentiation of NHBE cells incubated at clonal density (7). Thus, control of squamous differentiation of NHBE cells is via a different mechanism than is operative for epidermal keratinocytes. Our results using NHBE cells are also similar to the observations with proadipocytes except that the origin of the factors is the platelets, in marked contrast to the proadipocyte differentiation-inducing factors which are isolated from plasma.

In conclusion, platelet factor(s) specifically cause growth cessation and induce squamous differentiation when incubated when NHBE but not lung carcinoma cells. Platelets contain a multitude of compounds (15) including growth factors for human and murine fibroblastic cells (1, 15, 17) and migration-stimulating activities (19). However, the platelet-derived growth factor isolated and purified by Antoniades et al. (1) is not responsible for either inhibiting growth or stimulating migration of NHBE cells. Platelet lysate fractions are currently being evaluated to completely characterize the factor(s) responsible for inhibiting growth and inducing squamous differentiation of the NHBE cells.

ACKNOWLEDGMENTS

The authors wish to thank S. Aaronson and A. Gazdar for providing lung tumor cell lines; W. Pettis, I. Berezovsky, R. Pendergss, and J. Quintero for technical assistance; H. N. Antoniades for a gift of HPPDFGF; S. H. Yuspa and A. Gazdar for comments on the manuscript; and P. Richardson for typing the manuscript.

REFERENCES


DECEMBER 1983

5919
Fig. 1. Cellular morphology and cell-cell arrangements of NHBE cells incubated for 10 days in serum-free medium (a and c) and 8% BDS (b and d)-supplemented medium: a, cells have a prolate spheroid cellular morphology; scanning electron microscopy, ×300; b, cells in apposition depicting few desmosomal junctions and a low content of tonofilaments; transmission electron microscopy, ×3000; c, large, flat, closely adjacent cells; scanning electron microscopy, ×300; d, cells in apposition depicting perinuclear vacuoles, numerous junctional complexes, and extensive networks of intermediate filament bundles; transmission electron microscopy, ×300.
Platelet Factors and Epithelial Cell Differentiation

Fig. 2. Cellular morphology and cell-cell arrangements of NHBE cells incubated for 2 days in CPDGF (3 units/ml); a, large cells with numerous microvilli; scanning electron microscopy, × 300; b, cells have few mitochondria and numerous perinuclear vacuoles circumscribed by a band of short tonofilament bundles. The nuclei are highly lobular and nucleoli are rare. Transmission electron microscopy, × 3000.
Differential Control by Platelet Factors of Squamous Differentiation in Normal and Malignant Human Bronchial Epithelial Cells

John F. Lechner, Irene A. McClendon, Moira A. LaVeck, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/43/12_Part_1/5915

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.