Butyrate Induced Reduction of Tumor Cell Laminin Receptors

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ABSTRACT

Laminin, a glycoprotein of basement membranes, binds to the surface of cultured human pancreatic carcinoma cells (PANC-1). The binding is saturable, with a high proportion of specific binding, as determined by competition of labeled ligand with 80-fold excess unlabeled ligand. Pretreatment of the carcinoma cells with butyrate markedly reduces the amount of specific laminin binding. The butyrate effect on laminin binding is dose dependent and is observed at a concentration of butyrate which does not significantly reduce cellular protein synthesis or increase laminin production. Scatchard analysis indicates that butyrate reduces the total number of laminin binding sites without affecting the binding coefficient (Kd = 2 nm). The laminin receptor protein isolated from the PANC-1 cell extract has a molecular weight of approximately 70,000. After butyrate treatment the total amount of extractable laminin receptor protein is significantly reduced. Thus butyrate reduces laminin binding to PANC-1 carcinoma cells by a mechanism which involves decreased expression of laminin receptor proteins in the plasma membrane.

INTRODUCTION

Laminin, a glycoprotein localized in basement membranes, is involved in a variety of biological events, including cell growth and differentiation (1–3), morphogenesis (4, 5), cell migration (6), cell attachment (7, 8), and cancer metastases (9). Laminin binds with a binding coefficient of 2 nm. The laminin receptor isolated from human breast carcinoma or melanoma cells (10) has a molecular weight in the range of 67,000–70,000 daltons and binding coefficient (Kd = 2 nm). The laminin receptor protein isolated from the PANC-1 cell extract has a molecular weight of approximately 70,000. After butyrate treatment the total amount of extractable laminin receptor protein is significantly reduced. Thus butyrate reduces laminin binding to PANC-1 carcinoma cells by a mechanism which involves decreased expression of laminin receptor proteins in the plasma membrane.

MATERIALS AND METHODS

Laminin Ligand. Laminin was purified from the EHS tumor as described previously (17) and was stored frozen in phosphate-buffered saline. Lociation was performed by the enzyme beads method (Bio-Rad Laboratories, Richmond, CA) yielding a specific activity of 5–9 mCi/µg.

Cell Culture. Human pancreatic carcinoma cell (18) PANC-1 is a continuous human pancreatic tumor cell line initiated from a poorly differentiated adenocarcinoma. The cells were grown to confluence in a humidified 37°C incubator in an atmosphere of 95% air and 5% CO2. Flasks of confluent cells were split (1:2) 24 h prior to assay yielding actively growing cells, 50–70% confluent. For pretreatment with butyrate, an aliquot of 100 µm n-sodium butyrate (Sigma Chemical Co., St. Louis, MO) in Tris-buffered saline was added to 75-cm² flask containing complete media with attached cells for a final concentration of 5 µm. The flask was incubated for 12 h in a humidified 37°C incubator in an atmosphere of 95% air and 5% CO2. The viability of the cells and cell growth were monitored using a hemocytometer for counting with trypan blue exclusion.

Total Cell Extract Preparation. Total cell extracts were prepared from PANC-1 cells using a lysis buffer (50 mM Tris, 1 mM CaCl2, 3.0 mM MgCl2, and 0.15 M NaCl with 112.5 µg n-ethylmaleimide, 12.5 mg phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40 per 50 ml of buffer, pH 7.4) and mechanical agitation at 4°C. The lysate was

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807

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centrifuged in a Beckman L5-75S ultracentrifuge at 100,000 × g for 45 min at 4°C. The excess Nonidet P-40 was removed by treatment of the extract with SM2 BioBeads (Bio-Rad Laboratories, Richmond, CA). The supernate was used immediately or stored at -40°C.

**Binding Assays.** Cells were removed from tissue culture flask using 2 ml of EDTA solution (0.2 g EDTA/fiter of phosphate-buffered saline without Ca²⁺ and Mg²⁺) (Grand Island Biological Co.). The cell suspension was added to 20 ml of RPMI 1640 medium without fetal calf serum and centrifuged in a Sorvall RT6000 at 1,000 rpm for 5 min and the resulting pellet was resuspended in Tris buffer (50 mM Tris:5 mM MgCl₂:2.5 mM CaCl₂:0.15 wt NaCl, pH 7.4) for washing. Laminin binding assays on intact cells were conducted in a 1-ml suspension of cells (4 × 10⁶ cells/tube) in triplicates to which 125I-laminin was added to each tube to reduce nonspecific binding. The cells were centrifuged at 1000 rpm for 5 min, and the pellets were washed twice using 1-ml washes of cold 0.1% BSA (final concentration, 1%/tube) was added to each tube.

The solutions were aspirated, and filters were washed six times, 10 min per wash, and autoradiography.

**Scatchard analysis.** Specific binding was a high proportion of total binding (Fig. 1). Scatchard analysis (Fig. 1) was linear (r = 0.95), Results expressed are the mean of triplicate assays. A Scatchard plot of the specific binding data for the PANC-1 cells (bottom panel) was linear (γ = 0.95), yielding a Kd of 1.5 nm and a Bmax of 35 ng laminin bound per 10⁶ cells (approximately 50,000 binding sites/cell). B/F = Bound/Free × 10⁶.

**RESULTS**

**Laminin Binding.** Laminin binding to suspended PANC-1 carcinoma cells was saturable. Specific binding was a high proportion of total binding (Fig. 1). Scatchard analysis (Fig. 1) was linear with a Kd of 1.5 nm and approximately 50,000 receptors per cell. When the PANC-1 cells were treated with butyrate (5 mM) for 12 h the specific laminin binding was markedly reduced by more than 90%. Scatchard analysis indicated that butyrate treatment
Reduced specific laminin binding was observed at butyrate concentrations which did not significantly reduce incorporation of proline into TCA precipitable proteins (Fig. 3). Furthermore, laminin production by the cells was not significantly enhanced by butyrate treatment. Using the dot blot immunoassay a semi-logarithmic relationship existed between the reflectance density of the color reaction and the concentration of the laminin antigen standard. The slope of this curve was used to determine the relationship between the absorbance of the color reaction and the amount of laminin in the test samples. The amount of laminin present in the cell samples was less than 10 ng per 10^6 cells (Fig. 4). This is less than 30% of the total amount of laminin required to saturate all the laminin binding sites. Thus, butyrate did not significantly increase the production of laminin by the PANC-1 cells.

Laminin Binding Activity of PANC-1 Cell Extracts. PANC-1 cell extracts were studied for laminin binding activity. The extract was immobilized on a solid phase (nitrocellulose), iodinated and used for laminin binding assays. The data showed that butyrate reduced the Bmax of the binding relationship but not the slope (Fig. 2).

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REDUCTION OF LAMININ RECEPTORS BY BUTYRATE

Fig. 6. Extraction of the laminin receptor. The laminin receptor was isolated from the PANC-1 cell extract using laminin affinity chromatography. The bound fraction was eluted (arrow) with 0.1 M glycine-HCl, pH 3.5. After elution, the bound fraction migrated as a single band on polyacrylamide gel electrophoresis with the molecular weight in the range of 70,000 and retained its ability to bind laminin compared to the unbound fraction (inset).

Fig. 7. Effect of butyrate on laminin receptor content. Sodium dodecyl sulfate gel electrophoresis autoradiography demonstrated binding of solubilized 125I-labeled PANC-1 extract to immobilized laminin. The total labeled proteins which bound specifically and nonspecifically to the laminin sepharose were removed from the solid phase by denaturation, applied to each lane, and run with reduction. Only the M,70,000 component (arrow; lane A) was specifically reduced by competition (lane B). Lanes A and B represent PANC-1 extract without butyrate treatment. Competition with unlabeled extract is demonstrated in lane C. Lanes D and C represent PANC-1 extract after cell incubation with 5 mM sodium butyrate. Competition with unlabeled extract is shown in lane D. The M, 70,000 laminin binding component (arrow) shows marked reduction after butyrate treatment and is diminished by competition (left panel). Solid phase specific laminin binding activity of PANC-1 extract after butyrate treatment (lanes C and D) are shown (right panel). Unlabeled laminin at 60- and 300-fold excess was used as competitor. The relative decrease in specific binding of laminin to the PANC-1 extract after butyrate treatment (right panel) represents the loss of extractable receptor content. Bars, SD.

laminin binding activity more than three-fold compared to controls. The extract from PANC-1 cells was studied by gel electrophoresis. A reduction in the M, 70,000 laminin binding component was observed in the butyrate treated cells (Fig. 7, left panel). The isolated laminin binding component retained specific binding properties demonstrated by competition with unlabeled cell extract (Fig. 7, right panel). These results indicate that butyrate treatment reduces the total content of functional M, 70,000 laminin binding protein expressed in the PANC-1 cell.

DISCUSSION

The present study demonstrates the modulation of a tumor cell surface receptor for laminin by the differentiating agent butyrate. Butyrate is a naturally occurring short chain fatty acid which causes morphological and biochemical changes in both normal and neoplastic cells. Butyrate can promote or induce the biosynthesis of new proteins (19), inhibit DNA synthesis and cell proliferation (20), illicit pronounced hyperacetylation of histones (21), and augment or reduce cell surface components or various enzyme activities (22-24). Several physiological effects of sodium butyrate on human pancreatic tumor cell lines have been documented (15). At a concentration of 1 mM butyrate present throughout the growth period of 28 days, butyrate inhibited the formation of PANC-1 colonies in soft agar. Cell growth experiments using 1 mM butyrate over a 12-day period demonstrated an increase in the doubling time for the PANC-1 cell line with a reduction of the saturation density (cells/cm²). Binding of laminin to receptors on neoplastic and normal cells has been reported previously (10, 16). The binding to these receptors is rapid, saturable, and reversible (16). Binding of 125I-labeled laminin can be inhibited by unlabeled laminin; other unlabeled proteins fail to block binding of labeled laminin. These findings satisfy the major criteria for designation of ligand-binding structures as specific receptors (25). The laminin receptor protein has been isolated, and monoclonal antibodies have been produced against the human laminin receptor isolated from breast carcinoma tissue (25).

Very little is known concerning the factors which regulate the expression and exposure of the laminin receptor. The present system offers one avenue to explore the relationship between differentiation and laminin receptor expression. To our knowledge, this is the first report of the effect of butyrate on specific laminin binding. Studies in our laboratory using the same experimental conditions did not elicit a response for the human breast carcinoma cell line MCF-7 (data not shown); however this is not totally unexpected, since different cell lines may require some variation in the experimental conditions. The effect of butyrate on other receptor-ligand systems has been explored. A reduction similar to ours has been observed in K562 cells; butyrate has been shown to reduce the expression of transferrin receptors in these cells (24). However, the dosage of butyrate which reduced transferrin receptors also reduced the cell growth rate. Thus for this cell butyrate may be affecting receptor expression indirectly through its effects on cell proliferation, which is dependent on transferrin uptake. In the present PANC-1 model, doses of butyrate which caused marked reduction in laminin receptors were not accompanied by a significant reduction in cell growth rate. Thus the observed effect was not simply due to an effect on cell growth. Furthermore, butyrate reduced laminin receptor expression under conditions in which the total cell protein synthesis was not significantly reduced. Therefore, we can also conclude that butyrate was not blocking total protein synthesis which could indirectly reduce the expression of laminin receptors. Butyrate has also been shown to reduce the level of receptors in other cell lines. In human breast cancer cells both estrogen receptors (21) and progesterone receptors (27) and, in HeLa S cells, glucocorticoid receptors (28), have been reduced by treatment with n-sodium butyrate. However, it should be noted that the experimental conditions required to produce the effect seen depended on the cell line and the receptor-ligand studied.

Cells which express laminin receptors may also secrete laminin itself. Consequently the total number of specific laminin binding sites could be reduced if the cell secreted more laminin which
blocked its own receptors. It would not be unexpected if butyrate increased laminin secretion, since other differentiating agents such as retinoic acid can induce parietal yolk sac carcinoma cells to produce more laminin and type IV (basement membrane) collagen. However, in the present model butyrate had very little effect on laminin secretion. In Fig. 4, the data indicate that PANC-1 cells accumulate only a small amount of laminin. Even in the presence of butyrate the laminin content was slightly greater than 10 ng/10^6 cells. This amount would saturate only a minority of the large number of laminin receptors expressed on these cells. Secretion of laminin was therefore not the mechanism by which butyrate reduced the laminin receptors. These data were further substantiated using anti-laminin antibodies and standard indirect immunofluorescence techniques (data not shown). No appreciable increase in cell surface laminin content could be detected in butyrate treated cells compared to untreated PANC-1 cells. Since the butyrate effect was not obviously related to cell proliferation, general protein synthesis, or laminin production, we studied the content of laminin receptor protein in the cell extract. Detergent extraction of the PANC-1 125I-labeled lysate was used to solubilize the laminin receptor which was isolated using laminin affinity chromatography. The isolated receptor exhibited a molecular weight of approximately 70,000 and retained its ability to specifically bind laminin immunobilized on nitrocellulose. The amount of extractable 125I-labeled protein which bound laminin was significantly reduced in the treated cells compared to the controls. In addition the amount of M, 70,000 125I-protein which could be competed by crude unlabeled extract was significantly reduced after butyrate treatment. Therefore, butyrate can alter the amount of M, 70,000 receptor protein which binds laminin. It is conceivable that butyrate could alter only the laminin receptor binding function without reducing the total amount of receptor. This possibility is not supported by the data in Fig 2, which show that the total number of receptors was reduced by butyrate with no detectable effect on the receptor binding constant. We can conclude that butyrate reduces the content of laminin receptor in the PANC-1 cell plasma membranes. Whether such reduction is due to decreased biosynthesis of the receptor or increased degradation of the receptor remains to be determined. For example butyrate could potentially stimulate an enzyme activity which degrades the receptor in the cytoplasmic compartment. Butyrate could also affect the composition of the cell plasma membranes altering the topological distribution, insertion, or internalization of the receptor protein.

These possibilities are under investigation. We are now studying the fate of the receptor after binding which will aid in answering these and other questions. The mechanism by which butyrate causes its effect remains to be elucidated; whatever the ultimate mechanism, butyrate treatment of PANC-1 carcinoma cells provides a useful system for studying the function and structure of the laminin receptor and its relationship to tumorigenicity.

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