Suramin Inhibits Cell Growth and Glycolytic Activity and Triggers Differentiation of Human Colic Adenocarcinoma Cell Clone HT29-D4*

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Suramin, a drug used in the treatment of trypanosomiasis and onchocerciasis inhibits growth factor-induced mitogenesis. In the present report, we show that suramin inhibits the growth of human colonic adenocarcinoma cells HT29-D4 and rapidly induces their differentiation into enterocyte-like cells. As soon as 6 days after the addition of suramin (100 μg/ml) in the culture medium, the cells form a polarized monolayer of regular columnar cells with occluding junctions delimiting two distinct membrane domains (apical and basolateral) and an apical brush-border expressing alkaline phosphatase and sucrase-isomaltase. The process of differentiation is fully reversible when the drug is removed from the culture medium.

We also show that suramin inhibits both glucose consumption and lactate production so that the glycolytic activity of the treated cells is lowered by 42%. This observation would shed some light on the complex mechanisms involved during the induction of HT29 cell differentiation when glucose is removed from the culture medium.

The difficulty in developing reliable methods to allow the obtention of confluent epithelial cells from intestinal primary cell cultures compelled many researchers to use established tumoral cell lines as models for the study of intestinal functions (1–4). Among the available cell lines, the human colonic adenocarcinoma cells HT29 are probably the most fascinating system since they can be induced to differentiate into enterocyte-like cells by simply removing glucose from the culture medium (5).

Because of a significant heterogeneity of the HT29 cell population, several attempts have been made to isolate subpopulations (6) or clones (7–9) from these cells. The clonal cell line HT29-D4 we have selected by limit dilution technique undergoes a typical enterocytic differentiation in glucose-free, galactose-containing medium (8). Moreover, HT29-D4 cells grown in glucose-containing medium can be induced to differentiate without cell loss in glucose-free medium in the absence of a progressive adaptation, which is necessary for the HT29-18 cell clone (9, 10).

The mechanism by which the removal of glucose induced such a differentiation is still unknown.

Suramin, a drug used in the therapy of trypanosomiasis and onchocerciasis, has a renewal of interest following recent discoveries: 1) suramin protects T lymphocytes against in vitro human immunodeficiency virus infection (11), probably by inhibiting the reverse transcriptase of the virus (12), 2) the drug inhibits the binding of platelet-derived growth factor to its receptor and is able to dissociate the receptor-bound growth factor (13–15), and 3) suramin inhibits the growth-promoting effect of epidermal growth factor and serum (16).

The effect of suramin on growth factor functions held our attention, and we investigated the action of suramin on the growth and differentiation of the clonal HT29-D4 cells grown in the presence of glucose. In this report, we demonstrate that suramin inhibits cell growth and triggers differentiation of HT29-D4 cells without any toxicity at the drug concentration used. In addition, we show that suramin inhibits both the utilization of glucose and the production of lactate. To our knowledge, the effect of suramin on cell differentiation has never been reported before. This observation would shed some light on the mechanism by which glucose starvation induces HT29 cells to differentiate.

**Experimental Procedures**

**Materiels**—Suramin1 (obtained from Specia, Paris, France) was prepared as a sterile stock solution of 100 mg/ml in distilled water and stored at −20°C. Fluorescent antibodies against rabbit IgG were from Sigma. The antiserum raised against human placental alkaline phosphatase was kindly supplied by Dr. Magali Theveniau (Marseille, France). The sucrase-isomaltase antiserum was generously provided by Dr. Isabelle Chantret (Villejuif, France). Culture media and supplements were from Eurobio (Paris, France).

**Cell Culture**—The human adenocarcinoma cell line HT29 was cloned by limit dilution technique (8). HT29 and HT29-D4 cells (passage 10–20) were grown in Dulbecco’s modified Eagle’s medium containing 25 mM glucose and 10% fetal calf serum (FCS). Suramin was added in the medium to reach the required concentration.

**Immunofluorescence**—Cells grown on glass coverslips were fixed with 3% paraformaldehyde in Ca2+- and Mg2+-free phosphate-buffered saline for 15 min at room temperature. The antiserum against alkaline phosphatase was used at a dilution of 1:100 in phosphate-buffered saline containing 1% bovine serum albumin and the succrase-isomaltase antiserum at a dilution of 1:200. Incubations were performed at room temperature for 90 min. Fluorescein-conjugated anti-rabbit IgG antibodies were used at a dilution of 1:100.

**Electron Microscopy**—The cells were fixed in situ with 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4, for 2 h, washed overnight in the same buffer with 7.5% saccharose, postfixed in 1% sodium tetroxide, then dehydrated in ethanol, and embedded in Epon.

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<th>1 Hexa</th>
<th>2</th>
<th>3</th>
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<tr>
<td>sodium sym-bis(m-aminobenzyl-m-aminopropylbenzoyl-</td>
<td>1-naphthylamino-4,6,8-trisulphonate)carbamide.</td>
<td>2 The abbreviation used is: FCS, fetal calf serum.</td>
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Effect of Suramin on Enterocytic Differentiation of HT29-D4 Cells

Chemical Analysis—Glucose concentrations in the deproteinized culture media were determined enzymatically with a GOD-Perid kit from Boehringer. Lactate concentrations were measured with a Sigma kit (procedure 826-UV).

RESULTS

Suramin Inhibits Cell Growth and Induces Cell Differentiation— Cultures of HT29-D4 cells growing in glucose-containing medium supplemented with 10% FCS were exposed to increasing concentrations (0.1 ng/ml to 10 mg/ml) of suramin 48 h after seeding. The morphological changes were followed by phase contrast microscopy. Already 4 days after suramin addition, the morphology of the cells was altered for drug concentrations between 50 and 500 pg/ml (data not shown). Cell toxicity was observed above 500 µg/ml suramin. The concentration of 100 µg/ml was chosen in all subsequent experiments.

The growth curves (Fig. 1) demonstrate that the addition of suramin (100 µg/ml) in the culture medium entailed a significant inhibition of cell growth. HT29-D4 cells grown in the presence of suramin reached confluency at a much lower density than control cells (3.5 × 10^5 cells/cm^2 versus 8 × 10^5 cells/cm^2 for untreated cells). This phenomenon was observed for the same cells grown in glucose-free, galactose-containing medium (8). The cells could be subcultured in the presence of suramin without alteration of the acquired morphology or cell loss for several months.

After suramin addition, the cells in the monolayer became polygonal (mainly hexagonal) and well delimited, as compared to control cells that appeared as densely packed multilayers. Fig. 2 shows the typical cell morphology of HT29-D4 cells cultured in the absence (Fig. 2a) or presence (Fig. 2b) of suramin for 6 days.

At this time, the ultrastructural observation showed that the suramin-treated cells formed a polarized monolayer of columnar cells with an apical brush border facing the medium and mature junctional complexes that divided the cell membrane in two domains (Fig. 2d). The control cells, on the contrary, consisted of poorly organized multilayers in which cell-cell contacts were exclusively ensured by desmosomes (Fig. 2c).

For comparison, the effect of suramin was investigated on the noncloned HT29 cells. Suramin, at the same concentration used for HT29-D4 cells (i.e. 100 µg/ml), induced the appearance of numerous bright vacuoles which represent intercellular lumina (not shown). At the ultrastructural level, numerous apical microvilli were observed in these lumina (Fig. 3a) and, in some fields, facing the medium (Fig. 3b). The perfect cellular organization of suramin-treated HT29 cells was never obtained, probably due to cell heterogeneity.

The Effect of Suramin Was Reversible—HT29-D4 cells were allowed to differentiate in the presence of suramin for 4 days, then the drug was withdrawn, and morphological changes were followed by phase-contrast microscopy. Data from Fig. 4, a and b, show that the cells reverted to the undifferentiated phenotype within 5 days. In the same set of experiments, we
have investigated the effect of an additional amount of FCS on the differentiating effect of suramin. HT29-D4 cells were cultured for 4 days in the presence of suramin in the culture medium containing 10% FCS, and the culture medium was supplemented with FCS to reach a concentration of 20%. The cells were allowed to grow in the presence of suramin in this medium during 5 more days. The cells returned to the undifferentiated phenotype shown in Fig. 4, c and d.

Suramin Induced Segregation of Apical and Basolateral Domains—Monolayers of control and suramin-treated cells were enzymatically radioiodinated in situ in the presence of lactoperoxidase. Cells were lysed, and samples were submitted to polyacrylamide gel electrophoresis. Protein patterns revealed by Coomassie Blue staining of the gel were identical for control and suramin-treated cells (Fig. 5, lanes 1 and 2, respectively). In contrast, striking differences could be seen between the two patterns of the radioiodinated proteins obtained from control cells (lane 3) and suramin-treated cells (lane 4). Most of the radioiodinated polypeptides obtained from suramin-treated cells displayed a molecular mass greater than 66 kDa.

Cell Surface Expression of Brush-border Hydrolases Alkaline Phosphatase and Sucrase-Isomaltase in Suramin-treated Cells—The presence of typical enterocytic brush-border hydrolases alkaline phosphatase and sucrase-isomaltase was assessed by immunofluorescence studies using highly specific antisera. The fluorescence displayed by control HT29-D4 cells grown in the absence of suramin was diffuse, although significant with anti-alkaline phosphatase antibodies (Fig. 6a) and very weak with anti-sucrase antibodies (Fig. 6b). Six days after the addition of suramin in the culture medium, numerous clusters of strongly positive cells were visible with anti-sucrase antibodies (Fig. 6d). Furthermore, when these cells were cultured 6 more days in the absence of suramin, the typical apical sucrase labeling had disappeared (Fig. 6f). The presence of alkaline phosphatase on the apical membrane of suramin-treated cells was also demonstrated (Fig. 6c). The labeling was lowered after the withdrawal of the drug (Fig. 6e).

Suramin Decreased Glucose Consumption and Lactate Production in the Culture Medium—HT29-D4 cells were cultured in control medium for 48 h, then suramin was added. The concentrations of lactate and glucose in the culture media were measured every day after the addition of the drug. Data from Fig. 7, a and b, show that suramin significantly decreased the consumption of glucose and sharply reduced the production of lactate during cell culture. The glycolytic activity in both cell types was assessed by calculating the ratio: amount of lactate produced versus amount of glucose consumed. The amount of lactate produced was 22.8 ± 0.8 μmol/mg of protein/24 h in control cells 10 days after seeding and 9.0 ± 0.2 μmol/mg of protein/24 h in cells treated with suramin for 8 days (Table I). Since the consumption of glucose was estimated to be 19.1 ± 0.8 μmol/mg of protein/24 h, then 10 days after seeding, suramin-treated cells were cultured for 2 days in the absence of suramin, then 10 days in the presence of suramin (100 μg/ml).

DISCUSSION

This work presents morphological and biochemical evidences that suramin, a drug used in the therapy of trypanosomiasis and onchocerciasis, was an inhibitor of HT29-D4 cell growth and a potent inducer of cell differentiation. HT29-D4 cells grown in the presence of suramin (100 μg/ml) underwent within 4 to 6 days a typical enterocytic differentiation similar to the one obtained by growing these cells in the absence of glucose (8, 17), based on the following observations. (i) These cells formed an epithelial monolayer constituted of well organized hexagonal shaped cells. (ii) Two distinct membrane domains (apical and basolateral) delimited by intercellular occluding junctions and a well differentiated apical brush-border were observed by electron microscopy. (iii) The molecular mass distribution of the radioiodinated polypeptides was different between the two cellular populations. Only polypeptides of M, above 66,000 were radiolabeled with suramin-treated cells. These polypeptides represent the external apical proteins accessible to lactoperoxidase in agreement with similar data obtained with glucose-starved HT29-
by the use of specific antisera in fluorescence studies. (v) The process of differentiation was reversible after removing the drug from the culture medium, as demonstrated by microscope studies. Moreover, the expression of sucrase-isomaltase was abolished, and the expression of alkaline phosphatase slightly reduced as soon as 6 days after removing the drug from the culture medium.

In an attempt to explain how suramin acts on cell differentiation, we have measured its effect at the level of glucose consumption and lactate production. It appeared that suramin provoked a significant decrease in glucose utilization, even though one remains far from a complete glucose starvation. For example, at day 9 after suramin addition, glucose consumption in suramin-treated cells was only 40% lower than that of control cells. As expected, lactate production was also inhibited, except during the first 48 h after suramin addition where a slight increase in lactate production was measured, probably due to glycogenolysis. Suramin also had a strong effect on the metabolic fate of the glucose taken up by the treated cells. In control cells, 1.19 molecules of lactate were produced from 1 molecule of glucose. After 8 days of suramin treatment, this ratio fell to 0.69 molecule of lactate produced from 1 molecule of glucose, which corresponded to a 42% inhibition of the aerobic glycolytic pathway.

Suramin has been described to efficiently reverse transformation of human fibroblasts after simian sarcoma virus infection (16). It is proposed that suramin neutralized an externalized v-sis protein (the oncoprotein from Simian sarcoma virus) structurally related to platelet-derived growth factor that prevented the cells from undergoing transformation. The authors show also that suramin inhibits the growth-promoting activity of epidermal growth factor and serum. The effect of suramin on the process of differentiation of HT29-D4 cells could be explained by its property of inhibiting the action of a broad spectrum of growth factors either present in the serum or secreted by the cells themselves (18).

An efficient reversion of suramin-induced differentiation of HT29-D4 cells was obtained by the addition of 10% FCS after the establishment of the differentiated state. This experiment suggests that suramin acts at the level of growth factors. It is likely that external factors trigger or prevent cell differentiation, and that glucose consumption was the consequence of these effects. By interfering with external signals such as growth factors, suramin could elicit the effect on differentiation we have observed. This hypothesis is supported by recent observations demonstrating the relationship between growth factor stimulation and carbohydrate metabolism in cultured cells (19). In 3T3 cells, both glucose uptake and glycolytic flux (measured by lactate production) were stimulated by epidermal growth factor and insulin (20). Similar effects were elicited by insulin-like growth factor-I in rat adipocytes (21),

### Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Glucose consumption*</th>
<th>Lactate production*</th>
<th>Glycolytic activity*</th>
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<tr>
<td>HT29-D4</td>
<td>19.1 ± 0.8</td>
<td>22.8 ± 0.8</td>
<td>1.18</td>
</tr>
<tr>
<td>Suramin-treated</td>
<td>13.0 ± 0.2</td>
<td>9.0 ± 0.2</td>
<td>0.69</td>
</tr>
<tr>
<td>HT29-D4</td>
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* Values are mean ± S.D. (N = 3).

**Fig. 6. Immunofluorescence staining with antisera against alkaline phosphatase and sucrase-isomaltase.**

- a and b, untreated HT29-D4 cells; c and d, HT29-D4 cells cultured in the presence of suramin for 6 days; e and f, HT29-D4 cells grown in the presence of suramin for 6 days, then cultured without the drug for 6 more days. The cells were stained with anti-alkaline phosphatase antibodies (a, c, e) or anti-sucrase-isomaltase antibodies (b, d, f). Bar, 25 µm.

**Fig. 7. Effect of suramin on glucose consumption and lactate production by HT29-D4 cells.**

- ○, untreated cells; ♂, cells treated with suramin (100 µg/ml) from the 2nd day after seeding (arrows). The culture medium was precipitated with trichloroacetic acid and centrifuged, and the clear supernatant was used for glucose (A) and lactate (B) determinations. Results are expressed as means ± S.E. of triplicate of two separated experiments.

D4 cells (17). These results suggest a physical separation between the two domains of the plasma membrane of differentiated HT29-D4 cells. (iv) Suramin was demonstrated to induce apical expression of two typical brush-border hydrolases, namely alkaline phosphatase and sucrase-isomaltase,
and transforming growth factor-β increased the uptake of 2-
deoxyglucose in 3T3 cells (22). It is tempting to speculate that
such factors continuously stimulate the glycolytic activity of
HT29 cells and that this metabolic abnormality prevents the
cells from differentiating. At least conceptually, this situation
can be abolished by: (i) removing glucose from the culture
medium; or (ii) blocking the effect of serum or secreted growth
factors with suramin. It should be emphasized that, in both
cases, HT29 cells were induced to differentiate.

We are aware that the mechanism of action of suramin on
cell differentiation is far from being elucidated, but our orig-
inal observation gives us the opportunity to investigate in this
way.

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script.

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