Proliferation of the Human Colon Carcinoma Cell Line HT29: Autocrine Growth and Deregulated Expression of the c-myc Oncogene

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ABSTRACT

Human colon adenocarcinoma cells (line HT29) are able to proliferate in a defined (serum-free) medium containing no added growth factors; in such conditions, their doubling time is 3 to 4 days (on serum-coated dishes) or 2 to 3 days (on an autologous extracellular matrix) compared with 1 day in the presence of fetal calf serum. In the presence of suramin, a polyanion disrupting the binding of growth factors to their receptors, the incorporation of [3H]thymidine in serum-free cultures is reduced (27.0 ± 2.9% of control after 3 days of culture), suggesting involvement of autocrine growth factors in the autonomous proliferation of the cells. The expression of the proliferation-related oncogene c-myc was examined during various stages of growth and differentiation of the HT29 cells. The cellular contents of c-myc mRNA were similar in all experimental conditions studied: exponential phase; stationary phase; nondifferentiated as well as differentiated cells (by glucose deprivation); and also in serum-free medium containing or not suramin. An approximately 2-fold increase in the level of c-myc mRNA was observed in cells cultured for 3 days in suramin-containing medium and then incubated during 3 h in the absence of suramin (with or without 10% fetal calf serum). Southern blot analysis of the genomic DNA of HT29 cells did not reveal any rearrangement within the region containing the c-myc gene and the flanking sequences (~five kilobases upstream and ~three kilobases downstream). The c-myc locus was weakly amplified (four to six copies per cell). These results indicate that the c-myc gene expression in HT29 cells is deregulated and does not require growth factor stimulation. The deregulation of the c-myc gene may be related to the reduced growth factor requirement of the HT29 cell line.

INTRODUCTION

The HT29 cell line was originally isolated by Fogh and Trempe (1) from a human carcinoma of the colon. The HT29 cells have retained certain characteristics of the normal tissue, such as hormone receptors (reviewed in Ref. 2). Furthermore, under various culture conditions, HT29 cells can undergo a differentiation in polarized monolayers of mucus-secreting and/or absorptive cells (reviewed in Refs. 3 and 4). The HT29 cell line secretes various peptides (5), including factors stimulating the proliferation of fibroblasts (6, 7).

Several lines of evidence suggest that protooncogene products are implicated in proliferation and differentiation processes, and that their deregulation is involved in the development of tumors. In that way, the c-myc oncogene is expressed when cultured cells are stimulated to proliferate, and its inappropriate expression is involved in carcinogenesis (reviewed in Ref. 8). A high level of c-myc mRNA has been evidenced in colon cell lines including HT29 (9).

In this paper, we have studied the growth requirements of HT29 cells and the implication of secreted growth factors, together with the expression of the c-myc oncogene in respect to cell proliferation and differentiation. Our results indicate that the proliferation of HT29 cells is largely independent of exogeneous factors, but reduced in the presence of suramin, known to disrupt peptide hormone-receptor binding (10, 11), thus implying an autocrine mechanism. The cells express the c-myc gene in all conditions of culture (e.g., in the absence or in the presence of serum), probably as a result of altered transcription or RNA processing mechanisms rather than of gene rearrangement.

MATERIALS AND METHODS

Materials. Suramin was a gift of Bayer-Pharma, Puteaux, France. The c-myc construction used (PCV 108 myc-neo, gift of F. Rijswijk) was the cosmids PCV 108 (neo) (12) containing an insert (Xhol-EcoRI) of the two coding exons of the human c-myc gene. The Clal-EcoRI fragment of the c-myc gene (third exon; cf. 13) was labeled by Multi-prime (Amersham, Les Ulis, France) and used as a probe (specific activity, ~2 × 10⁶ dpm/ng of DNA).

Cell Culture. The human colon adenocarcinoma cell line HT29 (1) was routinely grown in Dulbecco's modified Eagle's medium containing 4.5 g of glucose/liter (Seromed, Intermed S.A., Toulouse, France) and supplemented with 10% fetal calf serum (Boehringer-Mannheim, Meylan, France). Differentiation was induced by culturing the cell in a medium without glucose and supplemented with galactose (1 g/liter); such cells displayed the morphological characteristics described (14, 15).

For serum-free culture, cells were harvested with trypsin-EDTA (Seromed) and the reaction was stopped with either soybean trypsin inhibitor (Sigma La Verpillière, France) or serum-containing medium. The cells were then washed in serum-free medium and seeded on either serum-coated dishes or autologous ECM (16) prepared as described by Bellot et al. (16). Briefly, HT29 cells were cultured until confluence in serum-containing medium. The cell monolayer was then washed with PBS, and the cells were allowed to disintegrate in PBS containing 0.5% Triton X-100. The lysate was discarded, and the dishes were rinsed 3 times with PBS. Extracellular matrix-covered dishes were then stored at −20°C until use. The SFM used was DMEM supplemented with glutamine (15 mM total), iron-saturated transferrin (15 to 30 μg/ml; Boehringer), and nonessential amino acids (Boehringer). For some experiments, HT29 cells were subcultured in serum-free medium for several passages on ECM and then transferred directly on plastic supports (without ECM). Such cells were referred to as HT29-S. This subline was maintained in a 50/50 mixture of SFM and Ham's F-12 nutrient mixture with glutamine at a final concentration of 24 mM. For the preparation of conditioned medium, cells were plated on dishes with ECM (HT29) or without ECM (HT29-S). The medium was changed after 24 h and conditioned medium was harvested 24 h thereafter for immediate use.

Cells were periodically tested for the presence of Mycoplasma with Mycotrim TC (Clinisciences, Paris, France) and were free of contamination.

Measurement of [3H]Thymidine Incorporation. Unless otherwise stated, 5 × 10⁴ cells per well were seeded on ECM in 6-well boxes or 35-mm Petri dishes (Falcon Becton-Dickinson, Grenoble, France). [3H]Thymidine (Amersham) was added (0.5 μCi/well) into 2 ml of medium obtained by mixing 1 ml of SFM and 1 ml of α-MEM (Gibco-BRL, Cergy-Pontoise, France) supplemented with glucose (total concentration, 4.5 g/liter), and incorporation was allowed to proceed for 2 days without medium change. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: ECM, extracellular matrix; SFM, serum-free medium; PBS, phosphate-buffered saline; SSC, standard saline citrate; DMEM, Dulbecco's modified Eagle's medium; α-MEM, α-minimal essential medium; SDS, sodium dodecyl sulfate; FCS, fetal calf serum; p21, M, 21,000 protein.
24 h. The reaction was terminated by adding 5 drops of 1 M ascorbic acid (17). The cells were washed twice with 1 ml of cold PBS, twice with 1 ml of cold 5% trichloroacetic acid (first acid wash included a 30-min incubation at 4°C), and solubilized in 0.6 ml of 0.1 N NaOH to which sodium dodecyl sulfate (0.1%) was added. A 0.5-ml aliquot of the solubilized extract was mixed with 5 ml of Aquasol scintillation fluid (Baker Deventer, Holland) and counted in an LKB liquid scintillation spectrometer.

Preparation and Northern Blot Analysis of RNA. The cells in 100-mm dishes were chilled on ice, washed once with cold PBS, and lysed with 1 ml of a solution containing urea (6 M) and LiCl (3 M) (18), to which SDS (0.05%) was added. The lysed cells were homogenized with Polytron and left overnight at 4°C. The homogenate was then centrifuged (30 min at 30,000 rpm in an SW-41 rotor), and the pellet was resuspended in 10 mM Tris (pH 7.6):1 mM EDTA:0.5% SDS:0.25 mM NaCl, deproteinized by two extractions with phenol/CHCl₃ and one extraction with CHCl₃, and precipitated with ethanol. Polyadenylated RNA was selected by chromatography on oligo(dT) cellulose (Sigma). RNA was denatured with 2.2 M formaldehyde in 50% formamide and 10 mM morpholine propanesulfonic acid (pH 7.0) for 10 min at 60°C; fractionated by electrophoresis in 1% agarose gel:80 mM Tris-phosphate (pH 7.5):2 mM EDTA. The DNA was denatured by soaking the gel in 0.5 M NaOH:1.5 M NaCl and then in 1.5 M Tris-Cl (pH 8), and 1.5 M NaCl was transferred onto a nitrocellulose filter with 3 M NaCl:1.5 M sodium citrate (20× SSC) and fixed by heating for 2 h at 80°C under vacuum. The nitrocellulose filters were hybridized with ³²P-labeled probe (labeled by Multiprime, Amersham) for 18 h at 42°C in 50% formamide and 2× SSC at 45°C and exposed to X-ray film, using intensifying screens (Du Pont Quanta III Chromex). The intensity of the signals was evaluated by densitometry.

Southern Blot Analysis of DNA. Fifteen µg of genomic DNA prepared from HT29 cells or from fresh human placenta were digested to completion with each enzyme 18 h at 37°C, with a 5-fold excess of the enzyme (and precipitated with ethanol. DNA was resuspended by incubation in 10 mM Tris-Cl:1 mM EDTA:0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, and 0.1% Ficoll. The filters were washed with 2× SSC at 45°C and exposed to X-ray film, using intensifying screens (Du Pont Quanta III Chromex). The intensity of the signals was evaluated by densitometry.

RESULTS

Growth of HT29 Cells in Serum-free Conditions. Several attempts have been made to grow HT29 cells in a defined medium (20, 21). In the absence of serum, attachment and spreading of cells are highly impaired (16). If the culture dish is coated with serum-containing medium and washed, HT29 cells are able to attach and spread (16). We have observed that HT29 cells grown on serum-coated dishes are able to proliferate in DMEM supplemented with nonessential amino acids, transferrin (15 to 30 µg/ml), and glutamine (14 mM total) (Fig. 1). Glutamine at a high concentration was an essential nutrient for HT29 cells (22), especially when cultured in defined minimal medium (not shown). In such conditions, after a lag phase (4 to 5 days) the HT29 cells are able to proliferate, although at a slower rate than in serum-containing medium (doubling time, 3 to 4 days) (Fig. 1, circles) versus 1 day in the presence of serum (not shown). When FCS is added during the lag phase, cells begin immediately to grow at the higher rate (squares). The cell density at saturation is 0.7 × 10⁶ cells/cm² and 1.4 × 10⁶ cells/cm² in SFM and in serum-containing medium, respectively. (It is to be noted that “saturation density,” the cells continue to divide and incorporate [³²P]thymidine (see further); cell detachment and death compensate for cell division, leading to a stagnation in cell number.)

When culture dishes are coated with serum, mitogens may be adsorbed to the plastic and slowly released into the medium (23), thus interfering with the culture system. In contrast with NRK rat kidney cells (24), HT29 cells did not attach in the presence of dithiothreitol-inactivated serum (not shown). Similarly, laminin and extracellular matrix derived from corneal endothelial cells were unable to promote attachment of HT29 cells (Ref. 16; Footnote 4). Therefore, we have used autologous extracellular matrix obtained after lysis of a confluent layer of HT29 cells previously cultured in serum-containing medium and subcultured for the first time in SFM on autologous extracellular matrix is shown in Fig. 1 (triangles). The doubling time was approximately 2 to 3 days, and the density of cells at confluence was 0.5 × 10⁶ cells/cm². In parallel, a subline of HT29 cells (HT29-S) has been established to grow without serum directly on plastic dishes after several passages on extracellular matrix. These cells (HT29-S) attach to plastic and grow in clusters without extensive spreading. Their growth is slightly faster than that of HT29 cells on ECM, but their saturation density is similar.³

Effect of Suramin on [³²P]Thymidine Incorporation. To test the possibility that the proliferation of HT29 cells in the defined medium may be due to an autocrine mechanism, cells were cultured in a defined medium in the presence of suramin. Suramin (200 µg/ml) inhibited [³²P]thymidine incorporation into DNA of cells on ECM (percentage of control, 27.0 ± 2.9, mean ± SEM of 10 different experiments). A 2- to 3-day

Fig. 1. Growth of HT29 cells in serum-free medium. Cells (10⁶/dish) were seeded in serum-free medium on dishes coated with FCS-containing medium. In one series of dishes (○), FCS (10%) was added to the medium on Day 4. A, 5 × 10⁶ cells/dish seeded in serum-free medium on dishes coated with extracellular matrix. Throughout the experiment, the medium in all dishes was refreshed every day or every other day. Cells were collected with trypsin/EDTA and counted with a hemocytometer. Points, mean of duplicates.

⁴ Unpublished observations.
³ M-E. Forgue-Laffite, unpublished observations.
treatment by suramin was needed to obtain maximal inhibition. Continuing the treatment for a further 24 h did not lead to an additional decrease in the \[^{3}H\]thymidine incorporation rate (not shown). Fig. 2, top, shows a typical experiment: \[^{3}H\]Thymidine incorporation in the presence of suramin (third day) is 21% of control. When suramin was removed from the culture medium on the fourth day, a 4-fold stimulation by serum (10% FCS) versus control medium (SFM) was obtained. This shows that suramin-treated cells remain sensitive to growth stimuli. Fig. 2, bottom, shows an experiment carried out with HT29-S cells (seeded without ECM). Similar results were obtained. \[^{3}H\]Thymidine incorporation was 12% of control in the presence of suramin (3 days), and the stimulation by serum was 3.6-fold. To test the effect of conditioned medium on \[^{3}H\]thymidine incorporation, cells pretreated with suramin (3 days) were washed and incubated (24 h) with the conditioned medium, harvested either from HT29 cells on ECM or from the HT29-S subline. The \[^{3}H\]thymidine incorporation was 84 and 74% of control cells (medium change), respectively. \[^{3}H\]Thymidine incorporation of serum-treated cells was -3-fold that of controls (not shown).

Expression of \(c\)-\textit{myc}. To further investigate the mechanism of growth deregulation in HT29 cells, the expression of the proliferation-related gene \(c\)-\textit{myc} was studied in various culture conditions. When cultured in DMEM containing 4.5 g/liter of glucose and supplemented with 10% FCS, HT29 cells grow as multilayers and are undifferentiated. When glucose is absent from the culture medium, replaced or not by galactose (1 g/liter), the cells undergo differentiation after confluence (14, 15). Northern blot analysis of polyadenylated RNA prepared from undifferentiated cells growing exponentially or in stationary phase and from terminally differentiated cells after confluence is shown in Fig. 3. The level of \(c\)-\textit{myc} mRNA is the same in all cases and is only decreased by 30 to 40% (Fig. 3, Lane C) when undifferentiated cells reach confluence. In addition, differentiated cells also express the \(c\)-\textit{myc} gene at confluence (Lane D).

The possible implication of autocrine growth factors in the expression of the \(c\)-\textit{myc} gene was investigated. A strong \(c\)-\textit{myc} signal was observed in total RNA prepared from cells cultured in SFM (Fig. 4A, Lanes 1). When cells were treated 3 days with suramin (Lanes 2), the expression of the \(c\)-\textit{myc} gene was not
notably altered, and a 24-h treatment with serum after 3 days of incubation with suramin did not enhance the c-myc mRNA level (Lanes 3). Since a transiently elevated expression of c-myc is frequently observed after exposure of cells to mitogens, we have analyzed RNA extracted from cells pretreated with suramin and then incubated during 3 h with either fresh (serum-free) medium or with 10% FCS. In the experiment shown in Fig. 4B, the c-myc mRNA contents in cells restimulated (after suramin) with SFM (Lane 2) or with 10% FCS (Lane 3) were, respectively, 1.6 times and 2.3 times that of the control cells maintained in suramin-containing medium (Lane 1).

Southern Blot Analysis of the c-myc Gene Region. To test whether the deregulation of the c-myc gene in HT29 cells was due to a modification of the gene, the genomic DNA extracted from HT29 cells was digested with EcoRI, PstI, and PvuII. The EcoRI fragment contains the whole c-myc gene (8 kilobases) and a 5-kilobase portion of the 5′-flanking region. The PstI and PvuII fragments encompassing the third exon of c-myc (6 and 2.5 kilobases, respectively) each contain a part of the 3′-flanking region (3 and 0.6 kilobases, respectively) (25, 26). Hybridization of the blot to the 32P-labeled probe of human c-myc showed no difference in the electrophoretic pattern between HT29 DNA restriction fragments and control DNA from human placenta (Fig. 5). However, the intensity of the signal detected with HT29 DNA was 2 to 3 times higher, indicating the presence of 4 to 6 copies of the c-myc gene per HT29 cell.

DISCUSSION

Previous reports have shown that, in serum-free medium, the HT29 cell line exhibits a weak dependence upon added growth factors (20, 21). Furthermore, the secretion of growth-promoting activities by HT29 cells suggested an autocrine loop (5–7). Our results (Fig. 2) show that HT29 cells, cultured on ECM in the absence of added growth factors, actively incorporate [3H]thymidine into their DNA. When cells were cultured in the presence of suramin, a sharp decrease in [3H]thymidine incorporation was observed. The presence of the drug for 2 to 3 days is needed to obtain maximal inhibition. It is possible that this period is necessary for all cells sufficiently advanced in the cell division cycle to complete the S phase; the growth curve (Fig. 1) showed a doubling time of 2 to 3 days for HT29 cells cultured in SFM. The inhibition of [3H]thymidine incorporation by suramin is not due to a toxic effect of the drug, since suramin-treated cells remain able to respond to serum. Cell growth in the absence of serum may depend on mitogens retained in the ECM. Alternatively, ECM itself may exercise a mitogenic or inhibitory effect on HT29 cells’ proliferation (16). In addition, the interaction with ECM could modulate the responsiveness of cells to growth factors (27). Further studies are necessary to elucidate this point. However, the fact that similar results were obtained with cells on ECM (Fig. 2, top) and the subline HT29-S (Fig. 2, bottom) cultured directly on plastic dishes rules out the possibility that serum factors adsorbed to ECM cause the proliferation of cells on ECM. Therefore, it may be concluded that the autonomous proliferation of HT29 cells is dependent, at least in part, upon the binding of factors synthesized by the cells.

The identity of autocrine growth-promoting factors for HT29 cells remains to be elucidated. Culoucas et al. (6) have purified two proteins secreted by HT29 cells that compete, respectively, with insulin-like growth factor 1 and epidermal growth factor for the binding to their receptors and that are immunologically related to them. However, the addition of anti-insulin-like growth factor 1 or anti-epidermal growth factor antibodies to the culture medium failed to inhibit the proliferation of HT29 cells (6), suggesting the existence of additional autocrine factors. Thus, Anzano et al. (7) have recently detected transforming growth factors α and β and platelet-derived growth factor-like material in medium conditioned by HT29. Our results showing an inhibitory effect of suramin on [3H]thymidine incorporation but no stimulatory effect of conditioned medium suggest other mechanisms. If autocrine factors are released into the medium, they may be unstable or rapidly degraded by the cells (for instance, after internalization). Alternatively, other ways of growth stimulation without secretion of the factor may be involved. This type of action may exist for factors like fibroblast growth factor (which lacks the hydrophobic leader sequence). Similarly, the int-1 oncogene product has never been detected in extracellular medium (reviewed in Ref. 28). It has been shown recently that growth factors expressed on the cell surface may act by cell-to-cell contact without being secreted; thus membrane-anchored pro-transforming growth factor α can bind to epidermal growth factor receptors of contiguous cells and induce autophosphorylation of the receptor, a rise in intracellular Ca2+ level, and an increase in c-fos mRNA. Several growth factors are derived from larger precursors with structural features characteristic of transmembrane forms. Such precursors are now believed to be active (reviewed in Ref. 29) before being cleaved and secreted. However, all of these stimulatory pathways imply the binding of either secreted or membrane-anchored factors to receptors of the target cell, a mechanism which is inhibited by the presence of suramin.

The continued, although reduced, incorporation of [3H]thymidine in the presence of suramin may be due to several reasons. Suramin may incompletely inhibit the binding of autocrine growth factors to their receptors. The maximal dissociation of 125I-labeled platelet-derived growth factor bound to plasma membranes of 3T3 fibroblasts was 85% in the presence of suramin (10, 11). On the other hand, in the case of transformed cells like HT29, a genetic independence of growth factors cannot be excluded, cells remaining stimulable by serum or autocrine factors but exhibiting a high “background” of prolif-
eration in a medium totally devoid of exogenous and endoge-
neous mitogens.

Under normal circumstances, the expression of the \textit{c-myc} protooncogene is induced when cultured cells are stimulated to proliferate, during embryogenesis and tissue repair. An inap-
propriate expression or a functional modification by mutation of the \textit{c-myc} gene is often involved in carcinogenesis and tumor development. Several molecular mechanisms have been evi-
denced in the deregulation of \textit{c-myc} expression in tumor cell lines; these include gene amplification, mutations, gene re-
arrangement, and chromosomal translocations. Constitutive expression of \textit{c-myc} alters the sensitivity of a cell to growth factors (8, 30).

The level of the \textit{c-myc} oncogene-encoded protein product was found to increase progressivly from normal colon mucosa through polyps to carcinomas (31). Accordingly, an ele-
vated expression of \textit{c-myc} occurs frequently in primary human colon carcinomas, and the regulation of \textit{c-myc} expression is altered in certain tumor-derived colonic cell lines (9, 32–34). The HT29 cell line exhibits a high level of \textit{c-myc} expression (9) in exponentially growing, stationary, nondifferentiated and dif-
f erentiated cells. The expression of \textit{c-myc} is only slightly re-
duced when undifferentiated cells reach confluence and slightly enhanced after a 3-h stimulation by (putative) autocrine factors or by serum. In that respect, the HT29 cell line differs from other human colon cancer cell lines (HCT 116 and MOSER), where differentiation/maturation agents induce a repression of \textit{c-myc} expression together with the reduction of cell prolifer-

Southern blot analysis of genomic DNA of HT29 cells and of human placenta led to the conclusion that a region of 16 kilobases encompassing the \textit{c-myc} gene, together with portions of 5-kilobase-upstream and 3-kilobase-downstream flanking se-
dquences, is not detectably rearranged in the HT29 genome. Although a point mutation or a translocation of a large segment including the gene cannot be excluded by the Southern blot, the data suggest a deregulation of the \textit{c-myc} transcription and/or a modification of the usually rapid turnover of the \textit{c-myc} mRNA.

In that respect, recent data indicate that the fusion of colon cancer cells having a deregulated \textit{c-myc} expression with nor-
mally regulated cells led to restoration of normal \textit{c-myc} tran-
script levels and control. The authors suggest the loss of a negative transregulatory element, suspected to be mapped on chromosome 59 near the locus of familial adenomatous poly-

The intensity of the \textit{c-myc}-hybridized band was 2 to 3 times higher in the DNA from HT29 cells than in normal DNA. This could be due to additional copies of the gene, not surprising in the polyheteroploid cells such as HT29 (1). The cells with a greater dosage of the \textit{c-myc} gene may gain a proliferative advantage. Trisomy of the chromosome 8 (bearing the \textit{c-myc} locus) is frequent in colon carcinoma; rearrangement of chromo-
some 8 have also been described in primary tumors of the large bowel (37, 38). A high level of the \textit{c-myc} transcript in human colon carcinomas (primary tumors and various cell lines) without amplification or rearrangement of the gene has been reported (32, 34). The deregulation of the \textit{c-myc} gene expression may contribute to the high proliferative capacity of HT29 cells and their low growth factor requirement (30). Al-
though an elevated constitutive expression of the \textit{c-myc} gene has been shown to be tumorigenic in certain cases (8), additional genetic changes may have occurred in the HT29 cell line.

However, when tested by focus formation assay in NIH-3T3 fibroblasts, the DNA extracted from HT29 cells lacked transfor-
mation activity (39, 40). Indeed, these cells have a normal but overexpressed p21\textsuperscript{WAF1} (39, 40); inhibition of the expression of the p21 by hypersomolality or by butyrate did not influence the HT29 cell cycle, suggesting that the overexpression of this protein was not the primary regulator of cell cycle progression in these cells (41).

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