Endocrine and mucous differentiation by a cloned human rectal adenocarcinoma cell line (HRA-19) in vitro: inhibition by TGF-β₁

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SUMMARY

Colorectal epithelium is composed of absorptive, mucous and endocrine cells, all of which are considered to arise from a common stem cell located in the crypt base. However, the factors controlling the commitment to differentiate are poorly understood. This is partly due to the lack of in vitro model systems for the study of differentiation in colorectal epithelium. The HRA-19 cell line, established from a human rectal adenocarcinoma, has been shown to have multipotential characteristics with cloned HRA-19 cells able to differentiate into absorptive, mucous and endocrine cells when grown as xenografts. The lack of such differentiated cells in HRA-19 monolayers in vitro suggests that differentiation is controlled by extracellular matrix, stromal cells and/or soluble factors. Such observations show that differentiation in HRA-19 cells can be controlled by extrinsic factors and therefore provide a model system for studying control of differentiation in colorectal epithelium. Unfortunately, the restriction of differentiation to xenografts of the cell line limits the degree to which this differentiation can be manipulated.

In this study, the possibility that HRA-19 cells could be induced to differentiate in vitro under appropriate conditions has been investigated. Endocrine and mucous cells were identified by immunocytochemistry with differentiation-related antibodies and histology of monolayers. Preconfluent HRA-19 cells grown in 10% foetal calf serum formed a well polarised monolayer with apical tight junctions and sparse microvilli, but cells with mucous or endocrine phenotypes were only very occasionally observed. However, endocrine and mucous cells could reproducibly be demonstrated in postconfluent monolayers grown in 1% foetal calf serum. The numbers of endocrine and mucous cells were quantified in monolayers, grown on coverslips, and this formed the basis for a differentiation assay. In this assay, TGF-β₁ inhibited HRA-19 cell proliferation and also inhibited differentiation into both endocrine and mucous cells.

Therefore, HRA-19 cells provide a useful model system for identifying factors controlling differentiation in colorectal epithelial cells.

Key words: differentiation, colorectal epithelium, TGF-β₁

INTRODUCTION

Normal colorectal epithelium is composed of absorptive, mucous and endocrine cells, which are thought to all originate from multipotential stem cells located at the base of the crypt (Chang and Leblond, 1971; Cheng and Leblond, 1974; Ponder et al., 1985). However the mechanisms which regulate commitment to the different cell lineages are poorly understood, partly due to the limited number of in vitro models of colorectal epithelial differentiation. Many cell lines have been established from human colorectal carcinomas and some of these retain differentiated features characteristic of the normal epithelium. These differentiated characteristics are usually of absorptive cells and, to a lesser extent, mucous cells. Although endocrine differentiation is a common feature of colorectal adenocarcinomas (Smith and Haggitt, 1984), only three colorectal carcinoma cell lines have been shown to express endocrine differentiation. In HRA-19 (Kirkland, 1986) and Caco-2 EJ6 (Caco-2 cells transfected with c-Ha-Ras) cells (De Bruine et al., 1993a), endocrine differentiation has only been demonstrated in xenografts. Endocrine differentiation was also observed in a limited number of Caco-2 xenografts obtained by inoculating cells in collagen gel because of the low take-rate of these cells. The only cell line described to date which displays endocrine differentiation in vitro is the NCI-H716 cell line (De Bruine et al., 1992). This line, isolated from the ascites of a patient with a poorly differentiated caecal carcinoma, grows as floating cell aggregates in vitro, with cells containing electron-dense granules characteristic of endocrine cells (Park et al., 1987). Further characterisation of this cell line showed that the cells could be undifferentiated or mucin-producing in addition to the endocrine phenotype, and that the endocrine differentiation could be markedly increased by xenografting. Recently endocrine differentiation in NCI-H716 cells has been shown to be enhanced in vitro by culturing cells in the presence of native extracellular matrix, fibroblast feeder layers and in serum-free
medium containing basic fibroblast growth factor (De Bruine et al., 1993b). In contrast to the NCI-H716 cells, endocrine differentiation in Caco-2 or Caco-2 EJ6 cells could not be induced in vitro even with extracellular matrix, feeder layers, or inclusion of basic fibroblast growth factor in the culture medium.

In our previous studies, endocrine and mucous differentiation of HRA-19 cells was restricted to xenografts. In this investigation, we studied the behaviour of cloned HRA-19 cells under a variety of culture conditions in an attempt to induce differentiation. Under certain in vitro conditions, differentiated phenotypes could be demonstrated in HRA-19 monolayers. This enabled the development of a differentiation assay using a cloned cell line which could be used to study the control of differentiation in colorectal epithelial cells.

TGF-β1 was tested for its effect on proliferation and differentiation of HRA-19 cells using this assay. TGF-β1 was chosen because many studies have indicated a role for this growth factor in control of growth and differentiation of colorectal epithelium. In addition, TGF-β1 has been shown to be a potent inhibitor of cell proliferation in other colorectal carcinoma and adenoma cell lines (Hoosein et al., 1987, 1989; Manning et al., 1991; Suardet et al., 1992).

This manuscript describes the development of a differentiation assay for colorectal epithelium using the multipotential HRA-19 cell line. The effects of TGF-β1 in this assay are described.

**MATERIALS AND METHODS**

**HRA-19 cell line**

The HRA-19 cell line was established from a primary human rectal adenocarcinoma (Kirkland and Bailey, 1986). Cloning of the cell line has previously been described (Kirkland, 1988a). Experiments in this study were performed with a cloned clone of the cell line designated HRA-19a1.1. between passages 20 and 30.

**Cell culture**

Cells were grown in Dulbecco’s Eagles Medium (Gibco, Paisley, Scotland) with 4,500 mg/l glucose. The medium was supplemented with 10% foetal calf serum (FCS) (Gibco), 100 μg/ml kanamycin and 110 mg/ml sodium pyruvate (BDH Chemicals). Confluent HRA-19 cells were trypsinised from T25 flasks and seeded into 6-well plates containing 22 mm × 22 mm glass coverslips. An overnight incubation in control medium allowed cells to attach before the experiment was started. This eliminated variation in cell attachment from experiments using different culture media. Cells were fed every 48 hours during the experiment. Coverslips were transferred to fresh wells if the medium became very acidic after 48 hours. Sufficient cells were seeded to allow them to become confluent 6 to 7 days after plating. At the end of the experimental period, cells were either fixed in ethanol for 10 minutes at room temperature for immunocytochemistry, or frozen at −20°C for DNA assay. Cell numbers were estimated using a fluorimetric DNA assay (Kirkland, 1985). Experiments were repeated at least three times.

Human recombinant TGF-β1 was obtained from Advanced Protein Products Ltd, West Midlands.

**Immunocytochemistry**

Immunocytochemistry was performed as previously described (Kirkland, 1988a), using monoclonal antibodies LK2H10 and PR4D4. LK2H10 (Boehringer) is an antibody recognising human chromogranin A and has previously been shown to be a specific endocrine tissue marker (Lloyd and Wilson, 1983). PR-4D4 is an antibody which reacts specifically with goblet cell mucous without columnar cell staining in colonic epithelium (Richman and Bodmer, 1987). These authors also demonstrated a very restricted pattern of staining with PR-4D4 in extracolonic tissues with the only staining being found in tracheal mucous-secreting submucosal glandular epithelium (Richman and Bodmer, 1987). Most colorectal carcinoma cell lines stained in their study were also found to be negative, although monolayers of the LS174T cell line, which produces mucous cells in xenografts, contained some positively staining cells (Richman and Bodmer, 1987).

**RESULTS**

Preconfluent monolayers of HRA-19 cells (days 0-7), grown in 10% FCS, formed a well polarized monolayer with apical tight junctions and sparse microvilli (Kirkland, 1986).

Fig. 1. Immunocytochemistry on intact monolayers of HRA-19a1.1. cells with antibody LK2H10. (A) Clustering of positively stained endocrine cells; (B) higher magnification of endocrine cells showing a cellular process (arrow).
However these preconfluent monolayers displayed negligible endocrine or mucous differentiation. Immunocytochemistry on these monolayers showed only a very occasional cell staining positively with either antibodies LK2H10 (human chromogranin) or PR4D4 (colonic mucous). However, postconfluent monolayers of HRA-19 cells contained both endocrine and mucous cells. The endocrine cells stained with the antibody LK2H10 to human chromogranin (Fig. 1A), and frequently displayed cellular processes which extended to surrounding cells within the monolayer (Fig. 1B). The endocrine nature of these cells was further confirmed by the demonstration of Grimelius-positive cells within the monolayer (Fig. 2). Mucous cells were demonstrated by positive staining with PR4D4 antibody (Fig. 3A); cells staining positively with PR4D4 were often large with distended cytoplasm (Fig. 3B). Alcian blue-positive cells were also present in HRA-19 monolayers (Fig. 4). Differentiated cells were not randomly scattered throughout the monolayer but frequently found clustered in patches (Figs 1 and 3). These patches contained both differentiated and undifferentiated cells and may represent clonal growth within the monolayer. The patchy nature of the differentiation necessitated assessment of the whole coverslip when counting the number of differentiated cells.

The differentiation observed in postconfluent monolayers was shown to be increased by decreasing the concentration of foetal calf serum in the culture medium (Fig. 5). A dose-responsive inhibition of both endocrine and mucous differentiation was observed with increasing concentration of FCS (Fig. 5). The concentration of FCS had little effect on proliferation, with average DNA values at 10, 5, 2.5 and 1% FCS being 76, 73.47, 74.4 and 67.86 μg per coverslip, respectively. Endocrine differentiation was found to be more sensitive to the inhibitory effects of FCS than mucous cell differentiation which was maintained at a significant level in 10% FCS. This illustrates differences in the control of endocrine and mucous differentiation of these cells. All further experiments were performed in 1% FCS.

Time course analysis of the number of differentiated cells showed increased differentiation with time, reaching a peak at 18 days and then declining (Fig. 6). This experiment confirmed our previous observations with 10% FCS, in that preconfluent monolayers contained few differentiated cells. The numbers of LK2H10- and PR4D4-positive cells were very low in day-4 monolayers. Following confluency at day 7, the numbers of
differentiated cells increased to a maximum at about day 18 (subsequent experiments showed good differentiation in day-20 cultures). By day 25 the number of differentiated cells had declined, probably due to a reduced cell viability in these monolayers. After 4 weeks without subculture, the culture medium becomes increasingly acidic and many floating cells are observed in the medium. The decline in differentiated cells in these monolayers suggests that differentiated cells have been lost from the monolayer, probably by detachment into the culture medium.

The effect of TGF-β1 on cell proliferation and differentiation of HRA-19 cells was determined by growing HRA-19 coverslips for 20 days in 1% FCS with varying concentrations of TGF-β1. Under these conditions, TGF-β1 was shown to inhibit cell proliferation of HRA-19 cells, with coverslips grown in 0.05 ng/ml TGF-β1 yielding only 62% of the DNA of control cells grown without TGF-β1 (Fig. 7). Little further inhibition was obtained with increasing doses of TGF-β1, coverslips grown in the presence of 1.85 ng/ml having 58% of the DNA of the control cells (Fig. 7). In addition, TGF-β1 was shown to be a potent inhibitor of both endocrine and mucous differentiation (Fig. 7). Staining with the chromogranin and PR4D4 antibodies was negligible in monolayers grown in TGF-β1 at 0.1 ng/ml and higher concentrations.

**DISCUSSION**

Cloned HRA-19 cells have multipotential characteristics and are able to differentiate into absorptive, mucous and endocrine cells in vivo (Kirkland, 1988a). In vitro, under standard culture conditions, differentiation is very limited. The two critical modifications which enabled us to reproducibly increase differentiation in vitro were long-term maintenance of confluent cultures and reduction of foetal calf serum (FCS) concentration.

Acquisition of differentiated characteristics in postconfluent cultures has previously been demonstrated in other human colorectal carcinoma cell lines. This phenomenon has been particularly well documented in the Caco-2 cell line which displays enterocytic differentiation in vitro, the differentiation process starting after confluency. In the Caco-2 line, alkaline phosphatase activity increases linearly with time after confluent growth.
Differentiation in colorectal epithelium

In this study, the differentiation observed in postconfluent cultures could be further increased by decreasing the concentrations of FCS to 1%. However the absolute level of differentiation was markedly altered by using different batches of foetal calf serum (unpublished observations), therefore serum-free conditions for this assay are currently being developed to eliminate such variability. The inhibitory effects of FCS on differentiation were possibly due, at least in part, to its content of TGF-\(\beta\), which was subsequently shown to inhibit both proliferation and differentiation of HRA-19 cells. However, as FCS did not inhibit proliferation, it is unlikely that sufficient TGF \(\beta_1\) was present to account for these findings. Many studies have supported a role for TGF-\(\beta\) in control of growth and differentiation in colorectal epithelium. Differential TGF-\(\beta\) staining has been demonstrated in both rat intestinal epithelium (Barnard et al., 1989) and human colonic epithelium (Avery et al., 1993), TGF-\(\beta\) expression being most abundant in terminally differentiated cells and least abundant in proliferative crypt cells. TGF-\(\beta\) has been shown to inhibit the growth of several well-differentiated colorectal carcinoma and adenoma cell lines; however many cell lines have been shown to be refractory to the growth inhibitory effects of TGF-\(\beta\) (Hoosein et al., 1987, 1989; Manning et al., 1991; Suardet et al., 1992). Interestingly, the conversion of an adenoma cell line to a carcinoma cell line has been shown to be accompanied by a decrease in response to TGF-\(\beta\) (Manning et al., 1991). This observation further supports the idea that loss of response to TGF-\(\beta\) may be an important event in the loss of growth control. As colorectal carcinoma cell lines have also been shown to secrete TGF-\(\beta\) (Coffey et al., 1987) and express TGF-\(\beta\) receptors (Coffey et al., 1987; Murthy et al., 1989), an autocrine growth inhibitory role for TGF-\(\beta\) has been postulated. Evidence in support of this hypothesis has recently been obtained as blocking antibodies for TGF-\(\beta_2\) have been shown to stimulate growth of Moser cells which are inhibited by exogenous TGF-\(\beta\) and also secrete TGF-\(\beta\) (Levine and Lewis, 1993).

In HRA-19 cells, TGF-\(\beta_1\) has been shown to be a potent inhibitor of cell proliferation, as expected for a well-differentiated cell line. This inhibition of cell proliferation is accompanied by an inhibition of both endocrine and mucous differentiation. These results are similar to those obtained with a rat jejunal crypt cell line (IEC-6) (Barnard et al., 1989) and murine keratinocytes (BALB/MK) (Coffey et al., 1988), where inhibition of proliferation was not accompanied by increased differentiation.

In addition to a direct effect on cells, TGF-\(\beta\) has also been shown to regulate cells by modifying interaction between cells and extracellular matrix. In colorectal carcinoma cells, TGF-\(\beta\) has been shown to increase laminin and fibronectin synthesis (Hoosein et al., 1987), regulate adhesion of cells to extracellular matrix (Chakrabarty, 1992), and increase glandular organisation of cells grown in collagen gels (Pignatelli and Bodmer, 1989). This latter effect was mediated by increasing expression of an integrin RGDT collagen receptor (Pignatelli and Bodmer, 1989). Therefore it will be important to study whether the inhibitory effects of TGF-\(\beta\) on the growth and differentiation of HRA-19 cells are maintained in the presence of extracellular matrix. The role of extracellular matrix itself is also of particular interest, following the report that the endocrine differentiation of NCI-H716 colorectal carcinoma

![Graphs showing dose-responsive inhibition of cell proliferation and differentiation of HRA-19 cells by TGF-\(\beta_1\).](image)

Fig. 7. Dose-responsive inhibition of cell proliferation and differentiation of HRA-19 cells by TGF-\(\beta_1\), (A) Mucous-positive cells; (B) chromogranin-positive (endocrine) cells; (C) total DNA content of culture. Mean ± s.d.
cells can be enhanced in the presence of native extracellular matrix (De Bruine et al., 1993b). Previous studies have demonstrated an important role for epithelial-mesenchymal interactions in gastrointestinal differentiation (Kedinger et al., 1986; Haffen et al., 1987). In our previous studies on HRA-19 cells, an increased absorptive cell differentiation was obtained by growing cells in collagen gels (Kirkland, 1988b). However, no evidence for endocrine or mucous differentiation was obtained under these conditions. This may have been due to the limited mucous and endocrine differentiation of cells grown in 10% foetal calf serum. The role of extracellular matrix molecules will be further clarified by investigating the appearance of differentiated phenotypes when HRA-19 cells are grown on different matrices. In addition, it will be important to determine whether HRA-19 cells secrete ECM molecules which play a part in controlling their differentiation.

The differentiation characteristics of the HRA-19 cell line will be used as a model of colorectal differentiation, with which to identify soluble factors and/or extracellular matrix molecules having a role in controlling the differentiation of colorectal epithelium.

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REFERENCES


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