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Cells obtained from colorectal microadenomas mirror early premalignant growth patterns *in vitro*

M. Richter[a], D. Jurek[a], F. Wrba[b], K. Kaserer[b], G. Wurzer[a], J. Karner-Hanusch[c], B. Marian[a,*]

[a]*Institute of Cancer Research, University of Vienna, Borschkegasse 8a, A 1090 Vienna, Austria*
[b]*Institute of Clinical Pathology, University of Vienna, Währinger Gürtel 18–20, A 1090 Vienna, Austria*
[c]*Department of Surgery, University of Vienna, Währinger Gürtel 18–20, A 1090 Vienna, Austria*

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Abstract

LT97, a permanent cell line consisting of epithelial cells with an early premalignant genotype was established from small colorectal polyps. LT97 cells have lost both alleles of the *APC* tumour suppressor gene. In addition, they carry a mutated Ki-*ras* oncogene, while *TP53* is normal. LT97 growth characteristics are thus representative of early adenomas. They had to be passaged as multicellular aggregates indicating a dependency of survival on cell–cell contact and in accordance with their premalignant genotype were not capable of growth in soft agar. LT97 cells did express both the EGF-receptor and small amounts of TGFα establishing an autocrine growth or survival pathway. However, in spite of autocrine TGFα production, growth was strongly dependent on exogenous growth factors—mainly EGF, insulin and HGF. Inhibition of the EGF-receptor kinase induced apoptosis at an IC_{50} concentration of 4 μmolar indicating that TGFα activated survival pathways in the early adenoma cell.

Keywords: Human colon; Adenoma; Growth control

1. Introduction

Tumour development in the colon and rectum is driven by the accumulation of genetic alterations in the tumour cells and accompanied by progressive deregulation of growth [1,2] via aberrant crypt foci (ACF) and premalignant adenomas [3,4]. Colorectal adenomas *in vivo* grow very slowly because they still contain a large fraction of resting cells and show a high incidence of apoptosis [5–7]. Compounds that modulate adenoma growth control can act either as stimulators or as inhibitors of colorectal carcinogenesis. Unfortunately, their slow growth characteristics make adenoma cells very difficult to propagate and study in culture and only a few established cell lines from adenomas at different stages exist until now [8,9].

While colorectal carcinoma cells easily grow in culture and many useful cell lines exist (e.g. [10,11], adenoma cells are difficult to maintain *in vitro* [8,9]. As a consequence, the mechanisms of growth regulation and survival have not yet been sufficiently investigated.

We have isolated early adenoma cells from the microadenomas of a patient suffering from hereditary familiar polyposis and established a cell line representing this early stage of tumour development that is the target population for tumour promoters. The cell line was characterised due to its genetic and growth characteristics and the effects of exogenous growth factors were analysed.

2. Materials and methods

2.1. Isolation of cells and initiation of cultures

Tissue specimens were obtained from the Department of Clinical Pathology within 1 h of subtotal colectomy. The specimen consisted of approximately 10 cm^2 of microadenoma containing mucosa, while the larger adenomas were retained for diagnosis of the disease stage.

* Corresponding author. Tel.: +43-1-4277-65241, Fax: +43-1-4277-9651.
E-mail address: brigitte.marian@univie.ac.at (B. Marian).

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The specimen was washed with a mixture of antibiotics containing 40 U/ml penicillin, 40 μg/ml streptomycin, 100 μg/ml gentamycin and 1 μg/ml amphotericin B, minced using two scalpels and then digested with 50 U/ml collagenase and 0.5 U/ml dispase in Minimal Eagle's Medium (MEM) containing antibiotics for 60 min at 37 °C. The epithelial aggregates were partially separated from fibroblasts and other single cell components of the digest by centrifugation at 1000 rpm for 60 s. The pellet of the epithelial cell aggregates was washed twice with MEM containing antibiotics and then plated in a small volume of growth medium consisting of MEM containing 2% fetal calf serum (FCS), 2×10^{-10} M triiodotyronin, 2 μg/ml transferrin, 1 μg/ml hydrocortisone, 5×10^{-9} M sodium selenite, 10 μg/ml insulin, 50 ng/ml fibroblast growth factor (FGF), 30 ng/ml epidermal growth factor (EGF) and 20 ng/ml HGF (MEM_{2+}) on plates coated with rat tail collagen.

For the initial eight passages, cells were detached using 10 U/ml collagenase. After the eighth passage, cultures were adapted to growth on uncoated tissue culture plates and the medium was changed to MCDB 302 containing 20% of L15 medium, 2% FCS, 2×10^{-10} M triiodo-L-thyronine, 1 μg/ml hydrocortisone (302 basic medium) supplemented with 10 μg/ml insulin, 2 μg/ml transferrin, 5×10^{-9} M selenite and 30 ng/ml EGF. Passaging was done using 5 mM ethylene diamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS) to detach aggregates of adenoma cells. The resulting culture population grew in monolayers of flat polygonal cells of epithelial origin as shown by their expression of cytokeratins (data not shown).

IEC18 normal intestinal epithelial cells, SW480, SW620 and HT29/HI1 carcinoma cells were kept under standard tissue culture conditions in MEM containing 10% FCS.

2.2. Growth fraction and apoptosis

Bromodeoxyuridine (BrdU) (5 μg/ml) was added to the growth medium for 48 h. Then, cultures were washed and fixed with methanol/acetone (1 + 1). BrdU incorporated in the nuclei was detected by immunocytochemistry using a commercially available detection kit (Amersham Pharmacia, Uppsala, Sweden).

Apoptosis was detected by staining parallel cultures with the Hoechst 33285 fluorescent dye. Nuclei with condensed or fragmented chromatin were counted as apoptotic.

2.3. Flow cytometry

Cells were trypsinised, washed twice with PBS and then incubated in nuclear isolation buffer (0.5 M citric acid, 0.5% Tween) for 5 min. Nuclei were released by moving through a syringe equipped with a 23-gauge needle, collected by centrifugation for 4 min at 2000 rpm and 4 °C and resuspended in 500 μl staining solution containing 50 μg/ml propidium iodide and 100 μg/ml RNAse A in PBS. A fluorescent activated cell sorter (FACS) Calibur (BD) was used for the flow cytometric analysis. It was equipped with a 15 mW argon laser exciting at 488 nm. The software used were CELLQUEST (BD) for data acquisition, and MOD-FIT for data evaluation. 25 000 events per analysis were stored in list mode files.

2.4. Anchorage-independent growth

IEC18 and SW620 cells were suspended in 0.3% agar noble in MEM plus 10% FCS, 20 U/ml penicillin, 20 μg/ml streptomycin and 0.1 μg/ml amphotericin B and poured onto a bottom layer of 1.5 ml of 0.7% agar noble in MEM plus 20% FCS, 40 U/ml penicillin, 40 μg/ml streptomycin and 0.2 μg/ml fungizon. For LT97 cells, agar was prepared using 302 medium and growth factors were added to the bottom agar in a 2-fold concentration. All cultures were seeded in quadruplicates at a density of 5×10^3 cells in six-well plates.

2.5. Exogenous growth factors

HGF was obtained from R&D Systems (Abington, UK), FGF from Paesel & Lorei (Duisburg, Germany). Parallel cultures of LT97 adenoma cells were shifted to basic 302 medium containing FCS, EGF, insulin, HGF or FGF as single growth factors. Cell number was determined 72 h later using neutral red uptake.

2.6. Isolation of genomic DNA

Genomic DNA was isolated from trypsinised cells by incubation with 0.2 mg/ml Proteinase K dissolved in 5 mM Tris containing 50 mM NaCl, 0.5 mM EDTA and 0.5% sodium dodecyl sulphate (SDS) solution for 2–4 h at 37 °C followed by extraction twice with 1 volume phenol and twice with 1 volume chloroform. DNA was precipitated with 2 volumes of isopropanol and redissolved in 1 ml TE-buffer (pH 8). 50 μl RNase A (1 mg/ml) was added for 30 min at 37 °C to remove the residual RNA.

2.7. Detection of mutations in Ki-ras

The polymerase chain reaction (PCR) was performed for the amplification of exon 1 using the 3′-primer 5′-ATT GTT GGA TCA TAT TCG-3′ and the 5′-primer 5′-ATG ACT GAA TAT AAA CTT GTG G-3′. The resulting DNA sample was sent for sequencing by MWG-Biotech AG (D-85560 Ebersberg) using the same primers.

2.8. Single strand conformation polymorphism analysis (SSCP)

Sequences covering mutation hotspots in Ki-*ras* and *TP53* were amplified by PCR using the following primers:

Sequence	Sense	Antisense	Product length (bp)
Ki-*ras* exon 1	tgt aaa cg acg gcc agt ttt tta tta taa ggc ctg ct	cag gaa aca gct atg acc gtc cac aaa atg att ctg aa	114
Ki-*ras* exon 2	tgt aaa acg acg gcc agt acc tgt ctc ttg gat att ct	cag gaa aca gct atg acc tga ttt agt att att tat gg	120
TP53 exon 4	atctac agt ccc cct tgc cg	gca act gac cgt gca agt ca	295
TP53 exon 5	gac ttt caa ctc tgt ctc ctt	acc agc cct gtc gtc tct ccg	253
TP53 exon 6	agg cct ctg att cct cac tga	cca gag acc cca gtt gca aac	170
TP53 exon 7	aag gcg cac tgg cct cat ctt	gca cagcag gcc agt gtg cag	192
TP53 exon 8	agg acc tga ttt tcc tta ctg c	tgc acc ctt ggt ctc ctc cac	231
TP53 exon 9	cct atc ctg agt agt ggt aa	cca aga ctt agt acc tga at	331

PCR products obtained from Ki-*ras* were run on 15% polyacryamide gels with 10% glycerol overnight at room temperature (codons 12/13) or 4 °C ambient temperature (codon 61), respectively. PCR products obtained from *TP53* were separated on 8–10% polyacrylamide gels containing 10% glycerol as described in Ref. [12]. Gels were stained using the Silver Stain Plus Kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.9. Detection of mutant p53 protein

Cells were homogenised in lysis buffer (50 mM Tris/HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA 20 μg/ml, 1% Triton-X100, 150 mM NaCl, 25 μg/ml aprotinin and leupeptin). 50 μg of soluble protein per lane were separated electrophoretically on 12.5% gels and transfered to PVDF membranes. Membranes were probed with the monoclonal antibody PAb421 (Calbiochem, San Diego, CA, USA) recognising both normal and mutant p53. Secondary antibodies were linked to horseradish peroxidase and detected using the enhanced chemiluminiscence Western blotting detection system (Pierce, Rockford, IL, USA).

2.10. Immunocytochemical staining

Cultures were washed with PBS, fixed with methanol–acetone (1:1, v/v) and stained using polyclonal antibodies against pan-cytokeratin (Dako, Glostrup, Denmark), monoclonal antibodies directed against the EGF-receptor (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), full length APC and the N-terminal segment of APC (Oncogene Research Products, Calbiochem, San Diego, CA, USA). Control preparations received non-immune serum. Binding was visualised using second antibodies coupled to biotin and avidin peroxide reagents (Vector Labratories, Burlingame CA, USA).

2.11. TGFα secretion

Cells were plated in 24-well plates and left to attach. 72 h later, the medium was changed and conditioned medium collected 24 h later. Transforming growth factor α (TGFα) secreted into the medium was determined by direct enzyme-linked immunosorbent assay (ELISA) using an assay kit obtained from Oncogene Research Products (Calbiochem). Cells were trypsinised and counted.

2.12. Effect of tyrphostin AG1478

Cells were plated in 24-well plates and left to attach for 72 h. After that, the medium was removed and 302 basic medium containing tyrphostin AG1478 diluted from a dimethylsulphoxide (DMSO) stock solution was added. Control cultures received corresponding amounts of DMSO. Cell number was determined by neutral red uptake at the end of the treatment period.

Cultures after removal of the neutral red solution were stained with Hoechst 33285 fluorescent dye to identify the apoptotic cells.

3. Results

3.1. Growth characteristics of LT79 adenoma cell cultures

To measure the growth fraction in LT97 cultures, cells were plated in full medium and left to attach for 4 days before the addition of BrdU for a further 48 h. Cells in S-phase were then detected using antibodies to BrdU. They comprised approximately 10% of the total population and were clustered together surrounded by a majority of BrdU-negative cells (Fig. 1a). Parallel cultures were shifted to basic medium containing only hydrocortisone, transferring and triiodotyronine (sfr) at day 4. This was not sufficient to maintain a constant cell number, and cell numbers decreased approximately 35% over 14 days (Fig. 1b). Addition of 30 μM EGF was the minimal condition necessary to balance cell production and cell loss. Addition of 2% FCS stimulated

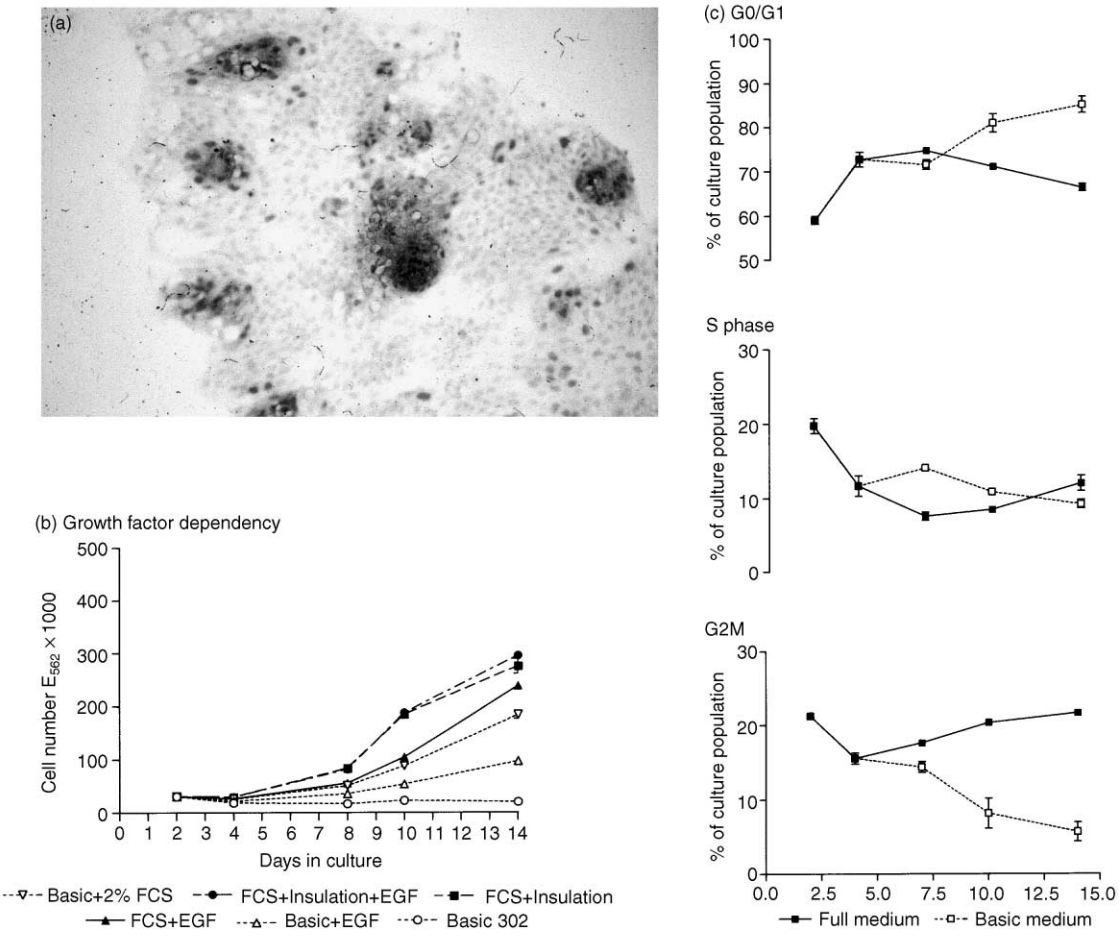

Fig. 1. Growth characteristics of LT97 cultures. (a) Cultures were incubated with 20 μg/ml BrdU for 24 h, washed and fixed. BrdU uptake into the DNA was detected after partial digestion with nuclease using a monoclonal antibody against BrdU, biotin-coupled second antibody and a peroxidase-coupled streptavidin reagents. (b) LT97 cells were plated in full medium and left to attach for 4 days. Thereafter, medium was switched to MCDB302 containing only hydrocortisone, transferrin and triiodotyronine (sfr), sfr containing 30 ng/ml EGF, sfr containing 2% FCS (basic 302), basic 302 containing EGF, basic 302 containing 10 μg/ml insulin and full medium (basic + EGF and insulin). Cell numbers were determined using neutral red uptake at the times indicated. The data represent three independent experiments (mean±standsard error of the mean (S.E.M.)). (c) Nuclei were isolated from LT97 cultures, incubated with propidium iodide and the cell cycle distribution analysed by flow cytometry.

growth and was additive to EGF. Addition of insulin (10 μg/ml) further stimulated cell growth (full medium, Fig. 1b and c).

Cultures kept in full medium maintained a fairly constant cell cycle distribution: over 14 days the G1 population increased only slightly from 59 to 66% at the expense of S-phase cells (20 and 12%, respectively). In basic medium, containing no additional factors, cells shifted into the G1 phase of the cell cycle mainly at the cost of the G2/M population, while the fraction of S-phase cells remained constant (Fig. 1c).

To investigate the effects of individual growth factors that had been essential for normal colorectal epithelial cells, these were diluted into basic 302 medium, added to parallel cultures and the cell number determined by neutral red uptake 72 h later. Full medium was used as a positive control. Among these growth factors, HGF was the most effective growth stimulator causing a 50%

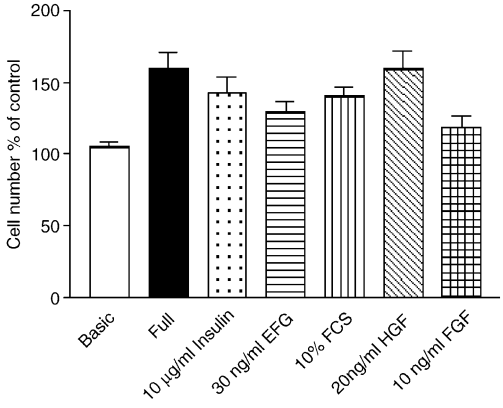

Fig. 2. Effect of individual growth factors on LT97 cultures. LT97 cells were plated in full medium and left to attach for 4 days. Thereafter, medium was switched to basic MCDB302 containing individual growth factors as indicated. Cell numbers were determined using neutral red uptake 72 h later. Results were calculated as a percent of controls kept in basic medium and represent the means±standard error of the mean (S.E.M.) from three independent experiments.

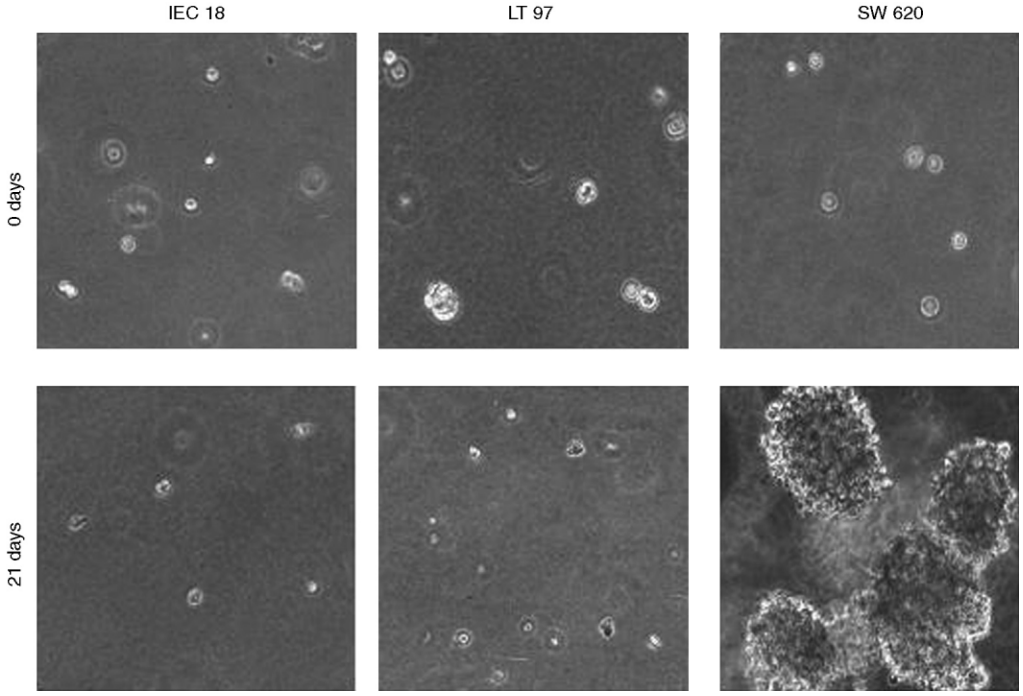

Fig. 3. Growth in soft agar. Cells were suspended in soft agar and incubated for 21 days. Growth was monitored twice weekly. While SW620 carcinoma cells grow to large colonies, no growth was detected in either IEC18 or LT97 cells.

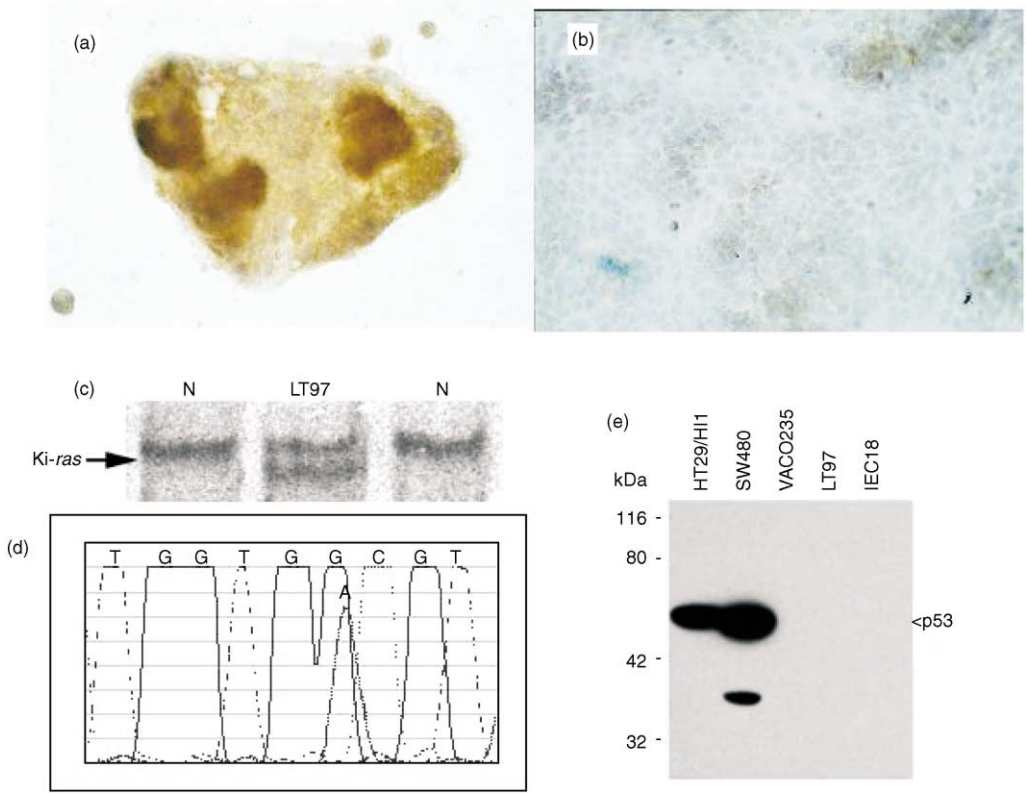

Fig. 4. Genotypic characteristics. (a, b) Cultures were stained using antibodies directed against the N-terminus and the C-terminus of the APC protein, respectively. (c) SSCP analysis of Ki-*ras* exon 1: the double band for LT97-derived PCR products indicates heterozygosity caused by mutation of one allele of the gene. Normal DNA (N) for controls was isolated from patient lymphocytes. (d) Ki-*ras* exon 1 was sequenced by standard PCR methods. (e) p53 protein was analysed by western blotting of total cell lysates from LT97 cells, HT29/HI1 and SW480 cells were used as positive controls and IEC18, T84 and VACO235 cells as negative controls.

increase in cell number similar to full medium (Fig. 2). Insulin and 10% FCS were slightly less effective, while EGF and FGF had only small effects on the cell number (Fig. 2).

The growth pattern and growth kinetics of LT97 cultures yielded no indication of a transformed phenotype. To ascertain this fact, cells were plated in soft agar. IEC18 normal intestinal epithelial cells and SW620 colorectal carcinoma cells were used as negative and positive controls, respectively. Like the IEC18 cells, and unlike SW620 carcinoma cells, LT97 cells were unable to grow under these conditions (Fig. 3). Aggregates present at day 0 died during the incubation period. The experiment was repeated using Roswell Park Memorial Institute (RPMI) medium as well as full 302 medium with identical results (data not shown).

3.2. Genetic characteristics of LT97 cells

As LT97 cells were obtained from a polyposis patient, a disrupted *APC* gene was expected. Using antibodies directed against either the N- or the C-terminus of the APC protein, reactivity could be detected against the former, but not the latter (Fig. 4a and b). Dark stained residues caught in densely packed areas of the culture only appeared after a prolonged reaction time and were also present in stains without the first antibody. This indicates that only a truncated form of the APC protein was present in the LT97 cells.

To analyse the genotype at the Ki-*ras* and *TP53* loci, SSCP analysis was performed using genomic DNA. The results showed that exon 1 of Ki-*ras* was heterozygous which indicates a mutation in codons 12 or 13 (Fig. 4c). To identify the exact mutation, exon 1 was sequenced which revealed a G⇒A mutation causing a Gly⇒Asp substitution at codon 13 (Fig. 4d).

TP53 exons 4–9 were homozygous (data not shown). To determine the p53 protein status, western blots were probed with monoclonal antibodies against the protein. Under such conditions, the normal protein is undetectable due to its short half-life and the presence of a protein band is indicative of a mutation. In contrast to lysates from SW480 or HT29 carcinoma cells, no p53 band could be detected in LT97 cells. VACO235 and IEC18 cells were used as negative controls (Fig. 4e).

3.3. Autocrine stimulation by TGFα

Colorectal carcinoma cells frequently secrete large amounts of TGFα that stimulate growth and survival pathways in an autocrine fashion [13–16]. The presence of the EGF-receptor was demonstrated by immunocytochemistry and showed a homogenous staining of all cells (Fig. 5a). TGFα secreted into the medium was measured by ELISA using SW620 cells as a positive control. The factor could be detected in the medium of LT97 cells, but at much lower concentrations than in the carcinoma cell line (Fig. 5b). When TGFα signals were disrupted by addition of the tyrosine kinase inhibitor tyrphostin AG1478 to the medium, cell loss was induced in a concentration- and time-dependent manner (Fig. 6a). The IC_{50} concentration after 48 h in three independent experiments was 4 μmolar. The incidence of apoptosis (AI) was determined morphologically after staining of the nuclei with Hoechst fluorescent dye. It was comparatively high already in control cultures (5%) which is characteristic for adenomas [7]. However, addition of tyrphostin to the culture increased the AI 7-fold to 35% indicating that cell loss was due to apoptosis (Fig. 6b and c).

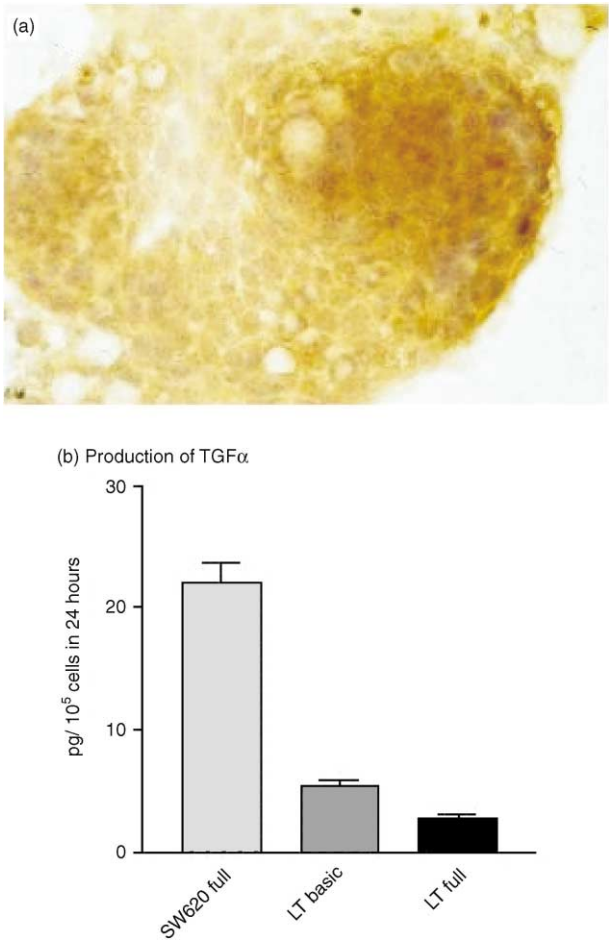

Fig. 5. TGFα and the EGF-receptor in LT97 cells. (a) Cultures were stained using a monoclonal antibody directed against the extracellular domain of the EGF-receptor, biotin-coupled second antibody and peroxidase-coupled streptavidin reagents. (b) 24-h conditioned medium was collected from triplicate cultures and the TGFα content measured by a direct ELISA. Cells were then trypsinised and counted. SW620 carcinoma cells were used as a positive control.

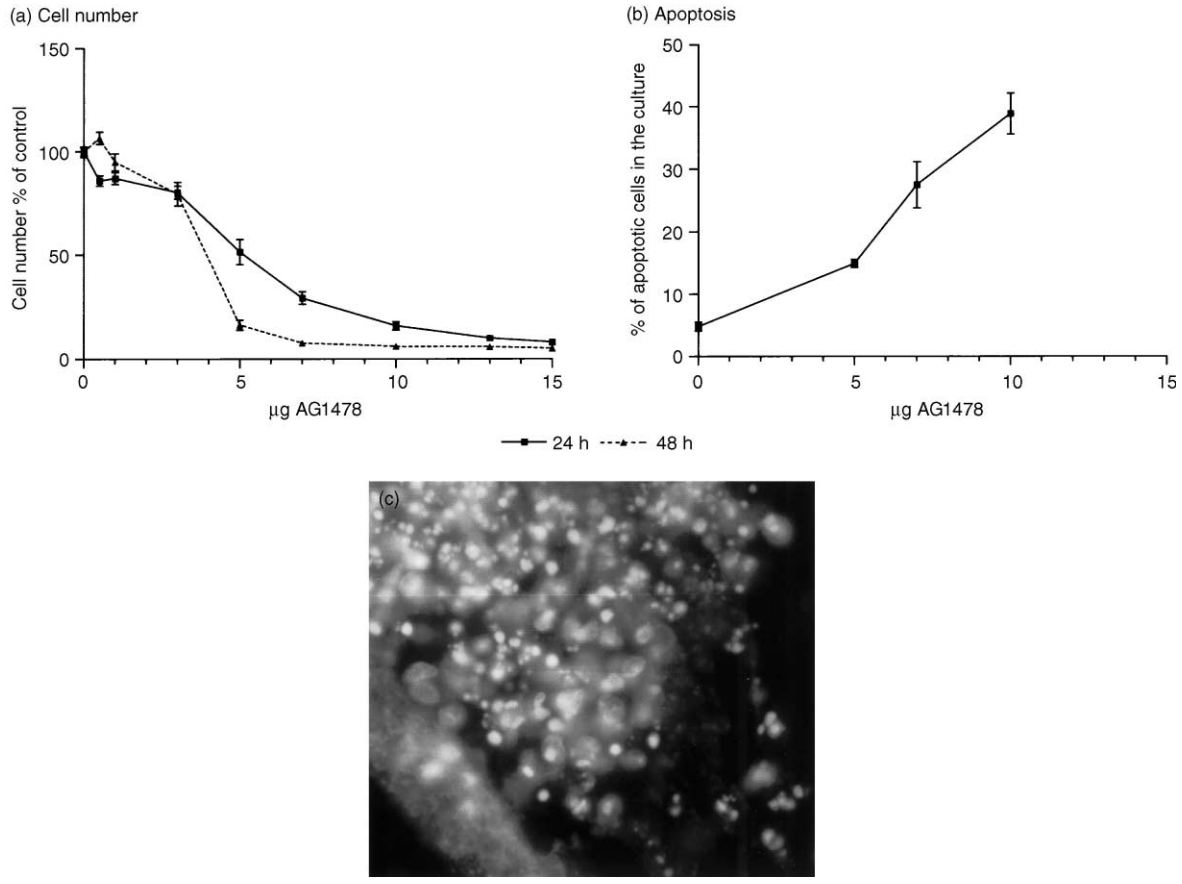

Fig. 6. Tyrphostin AG1478-induced cell loss. Cells were exposed to tyrphostin AG1478 diluted into basic 302 for 24 or 48 h: (a) Cell number was determined by neutral red uptake; (b) the fraction of apoptotic cells were determined by counting 1000 cells/well from triplicate cultures; (c) cultures after removal of the neutral red dye were washed and stained with Hoechst 33285 to determine nuclear morphology.

4. Discussion

The LT97 cell line reported here has been established from the microadenomas of a patient suffering from familial polyposis coli—a tissue representing the earliest possible premalignant stage from which tissue specimens can be obtained after surgery. The cultures have been maitained for up to 50 passages, but have not been cloned from single cells. This is due to the fact that single LT97 cells do not survive so that cultures have to be passaged as cell aggregates. Therefore, we have to assume that the population is not completely homogenous. However, all cells in the population express cytokeratin identifying them as epithelial cells. Similarly homogenous is the expression of the EGF-receptor which is characteristic of the colorectal epithelium.

The premalignant genotype is characterised by the presence of a truncated APC protein, as shown by the lack of reactivity against antibodies directed to the C-terminus of the protein in every cell of the culture population. Mutation in *APC* is the genetic defect that causes familial polyposis coli [17] and was therefore expected to be present in the adenoma cells obtained from a polyposis patient. Loss of the normal allele causing complete loss of full length APC from the cells is one of the most common genetic defects in colorectal tumour cells [2]. It occurs early in tumour development and has already been observed in aberrant crypt foci (ACF) that represent the earliest premalignant lesions in the gut [18,19].

In addition, LT97 had a mutated Ki-*ras* as has been shown in approximately 50% of colorectal tumours. This mutation has also been detected in ACF [18,19] and might confer an enhanced growth potential and resistance to apoptosis [20,21]. Propagation *in vitro* seems to select for cells carrying a *ras*-mutation as it could not be detected at the early passages (data not shown), but was clearly present later on. The wild-type status of p53 did not change during passaging. Together the genetic alterations detected in LT97 cells are characteristic of early premalignant colorectal tumour cells [2].

The growth characteristics of LT97 cells reflect a premalignant phenotype as shown by their growth factor dependency, as well as their incapability to grow in soft agar. Tumorigenicity in nude or severe combined

immunodeficient (SCID) mice was not investigated as it was assumed to be negative for cells that are not capable of anchorage-independent growth. Growth on tissue culture plastic is dependent on exogenous growth factors—specifically EGF and insulin, that stimulate proliferation and survival pathways [22]. When individual growth factors were tested, HGF was the strongest stimulator of growth. The factor has been described as a mitogen and morphogen in fetal gut [23]. EGF on the other hand had only minimal effects on cell number. In cultures of normal colorectal epithelial cells, it has been shown to be a survival factor for resting terminally differentiated cells [15]. This activity was still observed in the LT97 cells as EGF prevented cell loss from cultures.

Like in the *in vivo* situation, the Ki-*ras* mutation did not cause a growth factor independence. Neither was the amount of autocrine TGFα sufficient to stimulate the growth of LT97 cells. However, it did seem to work as a survival factor, as blocking of the EGFR-receptor kinase induced apoptosis. This was also observed in colorectal carcinoma cells, as well as in VACO235 adenoma cells that were obtained from a villous adenoma [14,24]. This reflects adenoma growth regulation *in vivo*, where the tissue localisation of TGFα was inversely correlated with apoptosis [25].

Lastly, LT97 do not express cyclo-oxygenase 2 [26]—a prostaglandin synthetase induced in larger adenomas—indicative of their origin from small microadenomas.

In summary, LT97 cells show genetic alterations characteristic for premalignant epithelial cells in the colon and have retained many of the growth characteristics of early adenoma cells *in vivo*. This makes them a suitable model to investigate mechanisms of tumour growth and progression and to test compounds modulating growth and gene expression in adenoma cells.

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References

1. Weinstein IB. Nonmutagenic mechanisms in carcinogenesis: role of protein kinase C in signal transduction and growth control. *Environ Health Perspect* 1991, **93**, 175–179.
2. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990, **61**, 759–767.
3. Bird RP. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Lett* 1995, **93**, 55–71.
4. Muto T, Bussey HJ, Morson BC. The evolution of cancer of the colon and rectum. *Cancer* 1975, **36**, 2251–2270.
5. Moss SF, Liu TC, Petrotos A, Hsu TM, Gold LI, Holt PR. Inward growth of colonic adenomatous polyps. *Gastroenterology* 1996, **111**, 1425–1432.
6. Kahl Rainer P, Sedivy R, Marian B. Protein kinase C tissue localization in human colonic tumors suggests a role for adenoma growth control. *Gastroenterology* 1996, **110**, 1753–1759.
7. Partik G, Kahl-Rainer P, Sedivy R, Ellinger A, Bursch W, Marian B. Apoptosis in human colorectal tumours: ultrastructure and quantitative studies on tissue localization and association with bak expression. *Virchows Arch* 1998, **432**, 415–426.
8. Willson JK, Bittner GN, Oberley TD, Meisner LF, Weese JL. Cell culture of human colon adenomas and carcinomas. *Cancer Res* 1987, **47**, 2704–2713.
9. Paraskeva C, Buckle BG, Sheer D, Wigley CB. The isolation and characterization of colorectal epithelial cell lines at different stages in malignant transformation from familial polyposis coli patients. *Int J Cancer* 1984, **34**, 49–56.
10. Leibovitz A, Stinson JC, McCombs WB 3rd, McCoy CE, Mazur KC, Mabry ND. Classification of human colorectal adenocarcinoma cell lines. *Cancer Res* 1976, **36**, 4562–4569.
11. Fogh J, Orfeo T, Tiso J, Sharkey FE. Establishment of human colon carcinoma lines in nude mice. *Exp Cell Biol* 1979, **47**, 136–144.
12. Kaserer K, Schmaus J, Bethge U, *et al*. Staining patterns of p53 immunohistochemistry and their biological significance in colorectal cancer. *J Pathol* 2000, **190**, 450–456.
13. Mendelsohn J. Epidermal growth factor receptor as a target for therapy with antireceptor monoclonal antibodies. *J Natl Cancer Inst Monogr* 1992, **13**, 125–131.
14. Partik G, Hochegger K, Schorkhuber M, Marian B. Inhibition of epidermal-growth-factor-receptor-dependent signalling by tyrphostins A25 and AG1478 blocks growth and induces apoptosis in colorectal tumor cells in vitro. *J Cancer Res Clin Oncol* 1999, **125**, 379–388.
15. Schorkhuber M, Karner Hanusch J, Sedivy R, *et al*. Survival of normal colonic epithelial cells from both rats and humans is prolonged by coculture with rat embryo colonic fibroblasts. *Cell Biol Toxicol* 1998, **14**, 211–223.
16. Coffey Jr. RJ, Goustin AS, Soderquist AM, *et al*. Transforming growth factor alpha and beta expression in human colon cancer lines: implications for an autocrine model. *Cancer Res* 1987, **47**, 4590–4594.
17. Bodmer WF, Bailey CJ, Bodmer J, *et al*. Localization of the gene for familial adenomatous polyposis on chromosome 5. *Nature* 1987, **328**, 614–616.
18. Smith AJ, Stern HS, Penner M, *et al*. Somatic APC and K-ras codon 12 mutations in aberrant crypt foci from human colons. *Cancer Res* 1994, **54**, 5527–5530.
19. Takayama T, Ohi M, Hayashi T, *et al*. Analysis of K-ras, APC, and beta-catenin in aberrant crypt foci in sporadic adenoma, cancer, and familial adenomatous polyposis. *Gastroenterology* 2001, **121**, 599–611.
20. Arber N, Sutter T, Miyake M, *et al*. Increased expression of cyclin D1 and the Rb tumor suppressor gene in c- K-ras transformed rat enterocytes. *Oncogene* 1996, **12**, 1903–1908.
21. Arber N, Han EK, Sgambato A, *et al*. A K-ras oncogene increases resistance to sulindac-induced apoptosis in rat enterocytes. *Gastroenterology* 1997, **113**, 1892–1900.
22. Wu X, Fan Z, Masui H, Rosen N, Mendelsohn J. Apoptosis induced by an anti-epidermal growth factor receptor monoclonal antibody in a human colorectal carcinoma cell line and its delay by insulin. *J Clin Invest* 1995, **95**, 1897–1905.
23. Brinkmann V, Foroutan H, Sachs M, Weidner KM, Birchmeier W. Hepatocyte growth factor/scatter factor induces a variety of

tissue- specific morphogenic programs in epithelial cells. *J Cell Biol* 1995, **131**, 1573–1586.
24. Richter M, Ebermann R, Marian B. Quercetin-induced apoptosis in colorectal tumor cells: Possible role of EGF receptor signaling. *Nutr Cancer Int J* 1999, **34**, 88–99.
25. Habel O, Bertario L, Adreola S, Sirizotti G, Marian B. Apoptosis and the tissue localization of TGF-alpha and the EGF-receptor in colorectal tumors. *Histochem Cell Biol* 2002, **117**, 235–242.
26. Richter M, Weiss M, Weinberger I, Furstenberger G, Marian B. Growth inhibition and induction of apoptosis in colorectal tumor cells by cyclooxygenase inhibitors. *Carcinogenesis* 2001, **22**, 17–25.