Abstract. Tributyrin (glyceryl tributyrate, TB) is known to induce malignant cells to differentiate followed by arrest of cell growth and death via apoptosis. We investigated the effects of TB on the distribution of cell cycle phases, differentiation as measured by alkaline phosphatase activity (ALP), and apoptosis of LS 174T colon cancer cells expressed by morphological changes, externalization of phosphatidylserine and stimulation of various caspases. TB (0.6 mM) reduced the proliferation by a 5-fold decrease of tumor cells in the S-phase and 1.3-fold increase in the G2/M-phase of cell cycle after 24 h of incubation. The ALP activity was enhanced in a dose-dependent manner up to 180-fold by 1 mM TB. Apoptosis was seen only above 0.6 mM TB (5-fold increase). Studies with caspase inhibitors revealed that TB mediated cell death was linked to up-regulation of caspases 3 and 8. Our results indicate that TB-induced differentiation promotes apoptosis in LS 174T cells and may explain the mode of action of TB finally resulting in an arrest of tumor cell growth.

Introduction

Tributyrin (glyceryl tributyrate, TB) is a prodrug of butyric acid known to exert growth inhibitory and differentiating effects on a wide variety of tumor cells in vitro (1-7). Compared with butyrate, TB reveals favorable effects on pharmacokinetic parameters without significant toxicity to patients with solid tumors (8-10). Antiangiogenic, anti-metastatic and apoptosis inducing effects were further postulated to explain the antitumoral activity of TB in various in vitro and in vivo models (4-7,11). Additional studies are ongoing to determine if a combination of butyrate analogs with other modalities such as immunomodulation or studies are ongoing to determine if a combination of butyrate and chemotherapeutics can improve the therapeutic potential (12-14). We have previously demonstrated, that TB in combination with IL-2 and IL-12 effectively increased the sensitivity of LS 174T colon cancer cells to NK cell-mediated cytotoxicity in vitro (15). Treatment with TB induced a more differentiated phenotype of LS 174T cells as reflected by the expression of various cellular adhesion molecules. We found a direct correlation between enhanced tumor cell killing by human NK cells and the induction of Fas and FasL by TB and IL-2/IL-12, respectively.

However, the molecular mechanisms by which butyrate and its derivatives lead to a differentiated phenotype of tumor cells are still poorly understood. Several studies proposed modulation of nuclear functions such as gene expression, acetylation of histones with altered chromatin conformation and DNA cleavage of internucleosomal regions that are typical for apoptotic cells (4,16). Other targets of butyrate are intracellular enzyme activities, cytoskeletal elements and external matrix proteins (17). It is well established that dysregulation of tissue homeostasis between proliferation, terminal differentiation and apoptosis play a pivotal role in colonic tumorigenesis. Malignant cells are usually characterized by a significant loss or blockade of normal patterns of cellular differentiation and diminished ability to undergo apoptosis (18). Therefore, agents that induce tumor cells to differentiate may promote both growth arrest and apoptosis. Effects of TB on growth restriction and apoptosis have been shown on human prostate cancer cells and MCF-7 mammary carcinoma cells (4,7). A more differentiated phenotype of either androgen-sensitive and androgen-resistant prostate cancer cell lines was found in agreement with an increased apoptosis in a dose-dependent manner up to 5 mM TB (7). On MCF-7 cells which show a functional lack of caspase 3, TB induced a time-dependent disruption of the mitochondrial membrane potential, expression of bax proteins and a caspase 3-independent cleavage of poly(ADP-ribose)-polymerase (PARP) (4). Caspase 3 is a key protease that becomes activated during the early stages of apoptosis (19). Proteases and their activators are important mediators of cell death and suggest novel therapeutic targets to treat malignancies (20).

The present in vitro study was designed to investigate the role of TB-induced differentiation on growth regulation and apoptosis of LS 174T colon cancer cells. Our results demonstrate, that TB induces apoptosis of more differentiated LS 174T cells as indicated by morphological changes, loss of phospholipid membrane asymmetry and involvement of proteolytic caspases. Our findings may contribute to the
understanding of the mechanisms of differentiation inducing effects of TB in the development of strategies to control the growth of human colon cancer cells.

Materials and methods

Materials. RPMI 1640 culture medium was obtained from Gibco BRL (Eggenstein, Germany), fetal calf serum (FCS) from Biochrom (Berlin, Germany), penicillin/streptomycin, trypsin/EDTA and the alkaline phosphatase kit were from Roche Diagnostics (Mannheim, Germany) and Lipofundin MCT® 10% from B. Braun Melsungen AG (Melsungen, Germany). Tributyrin (TB) and propidium iodide were purchased from Sigma (Taufkirchen, Germany). The annexin V apoptosis detection kit, caspase 3 colorimetric assay and caspase inhibitor sample pack were from R&D systems (Wiesbaden, Germany). The nucleosome EIA was from Calbiochem-Novabiochem (Frankfurt, Germany) and the BCA assay from Pierce (Rockford, IL).

Cell culture. LS 174T human colonic adenocarcinoma cells (kindly provided by Dr I. Fichtner, MDC Berlin-Buch, Germany) were grown in RPMI 1640 medium containing 10% (v/v) heat-inactivated FCS and 0.5% (v/v) penicillin/streptomycin (21). Cells were routinely cultured in 25 cm² plastic culture flasks at 37˚C in a humidified atmosphere of 5.5% CO₂ and passaged twice weekly with 0.05% trypsin containing 0.02% EDTA when they reached confluency. Routine assays for mycoplasma were negative. For in vitro studies, tributyrin (98% pure by gas chromatography) was emulsified in Lipofundin MCT 10% to yield a 20% (v/v) homogeneous stock solution as described (3). To guarantee stability, the emulsion of tributyrin was freshly prepared for each experiment and serially diluted in standard medium before addition to the cell cultures.

Cell cycle analysis. Single cell suspensions obtained from monolayer of LS 174T cells were washed in ice cold phosphate-buffered saline (PBS), fixed with methanol and incubated at -20°C for 30 min as described (22). The cells were then resuspended in 500 µl RNase type II A (2 mg/ml PBS) and stained with 250 µl propidium iodide (100 µg/ml PBS) at room temperature for 30 min before measuring the DNA content using FACSCalibur® flow cytometer (Becton Dickinson, Heidelberg, Germany). The fluorescence intensity of 10,000 events were analysed to quantify the percentage of cells in the A, G0/G1, S, and G2/M-phases of the cell cycle. Data were acquired and evaluated using CellQuest™ software (Becton Dickinson, Heidelberg, Germany).

Assay of alkaline phosphatase activity. To determine the differentiating inducing effects of tributyrin, the activity of the membrane-associated enzyme alkaline phosphatase (ALP) was measured using para-nitrophenylphosphate as substrate at pH 9.5 and 37°C (23). The extent of hydrolysis was spectrophotometrically determined at 405 nm using an UVIKON 930 photometer (BioTeck Instruments, Neufahrn, Germany). Results were expressed as mU per mg of cellular protein according to the BCA method (24). The cell lysates were frozen at -50°C, thawed and agitated for 5 min prior to the alkaline phosphatase activity and protein determinations. Each value represents the average of four experiments with triplicate measurements.

Apoptosis. The cells were evaluated for evidence of apoptosis by different assays: i) measurement of cellular DNA content by propidium iodide staining and FACS analysis to detect a subdiploid peak indicative for DNA fragmentation, ii) pappenheim staining to identify morphological characteristics of apoptotic cells (25) and iii) translocation of phosphatidylserine on the outer plasma membrane by flow cytometry (26). In these experiments, 5x10⁶ LS 174T tumor cells were seeded in 24-well microtiter plates, incubated overnight and treated with different concentrations of tributyrin for up to 6 days. Culture medium was changed at first on day 3 and then daily in all experiments. For morphological analysis cells were transferred to glass slides using a cyto-centrifuge (Shandon, Frankfurt, Germany), pappenheim stained, air-dried and photographed at x200 magnification using Kodak 100 film.

Caspase 3 activity. The activity of caspase 3 was quantified using a commercially available caspase 3 colorimetric assay (R&D Systems GmbH, Wiesbaden, Germany) in which the proteolytic cleavage of the chromophore p-nitroanilide (pNA) from the labeled substrate DEVD-pNA was spectrophotometrically detected at 405 nm on SLT Easy Reader (19). Adherent cells were harvested by trypsination at different times, lysed and processed according to the manufacturer's instructions.

Caspases inhibitor studies. For selected experiments, cells were preincubated with different peptide inhibitors of caspases 3, 8, and 9 at the concentration of 100 µM (R&D Systems, Wiesbaden, Germany) 24 h before treatment with 0.6 mM TB for additional 3 days. Free nucleosomes in apoptotic cell extracts were measured by a non-isotopic Nucleosome-EIA kit (Calbiochem-Novabiochem, Frankfurt, Germany) with lyophilized standards designated as nucleosome units.

Statistical analysis. The data from at least three independent experiments were calculated for statistical differences between treated and untreated tumor cells utilizing the Mann-Whitney U test for non-paired samples with a probability value of less than 0.05, considered to be significant. The evaluations were performed with the GraphPad software Version 1.15.

Results

Effects of TB on cell cycle phases of LS 174T cells. To explore the mechanisms responsible for the antiproliferative effects of TB on LS 174T cells we assessed the cell cycle distribution after staining with propidium iodide and flow cytometric analysis. This method also allows to quantify low molecular weight fragmented DNA related to the number of cells undergoing apoptosis. Exposure of exponentially growing LS 174T cells to 0.6 mM TB for 24 h resulted in a marked loss of cells in the S-phase from 25 to 3.5% and a significant increase of cells in the G2/M-phase from 23 to 30% (Fig. 1). No changes were observed after 8 h of incubation. The
The proportion of apoptotic cells rose from 1.6 to 5.1%, whereas cells in the G0/G1-phase were only slightly augmented. Previous studies have shown that TB inhibits the proliferation in a dose-dependent and reversible manner with an IC50 value of 0.45 mM after incubation for 6 days. Changes in the cell cycle distribution were time-dependent between 8 and 72 h. A significant decrease of cells in the S-phase began after 20 h and was complete after 72 h in the concentration range between 0.2 and 1 mM TB. The accumulation of cells in the G2/M-phase was only significant with concentrations greater than 0.6 mM TB. Approximately 24 h after treatment with 0.6 mM TB a fraction with subdiploid DNA content was detected followed by a further increase (data not shown).

Effects of TB on differentiation of LS 174T cells. To determine the differentiation inducing effects of TB on LS 174T cells the ALP activity was studied over a 3-day period. The cells showed a low baseline enzyme activity of 0.413 mU/mg of cell protein. TB was found to be a potent inducer of ALP activity in a dose-dependent manner (Fig. 2). Incubation of the cells with TB at 0.25 and 1 mM augmented the enzyme activity to 4 and 75 mU/mg of protein, respectively, corresponding to a 10- and 180-fold increase relative to control. An EC50 value of about 0.8 mM TB was calculated.
with a plateau phase starting at 1 mM TB. Incubation with TB for more than 3 days did not further enhance the ALP activity significantly. Removal of TB 3 days after addition caused progressive reduction in enzyme activity (data not shown).

**Effects of TB on the apoptosis.** The apoptosis inducing effects of tributyrin on LS 174T cells were followed by morphological characteristics using pappenheim staining and detection of phosphatidylserine by the annexin V-FITC method. The cells underwent typical apoptotic changes by morphological criteria upon treatment with 0.6 mM TB. Cytoplasmic condensation demonstrated by cell surface blebbing and nuclear fragmentation were observed after 3 days. When untreated LS 174T cells were cultured in tissue flasks, apoptotic features were seen after 6 days when some of the cells detached and floated in the medium. One of the earliest characteristics of apoptosis is the translocation of phosphatidyl-serine on the outer plasma membrane, binding with high affinity to annexin V. As illustrated in Fig. 3, TB stimulated apoptosis as percentage of cells staining positive for annexin V-FITC in a dose and time-dependent manner. The number of apoptotic cells were significantly enhanced in the adherent population after 3 days of incubation with 0.6 mM TB. Treatment for 6 days caused a 5-fold increase from 7 to 37%. Loss of membrane integrity obtained with 0.2 mM TB over a 6-day period was not significantly different from untreated control.

**TB-induced activation of caspase 3.** Treatment of LS 174T cells with TB resulted in an activation of caspase 3 as measured by the proteolytic cleavage of the peptide substrate DEVD-pNa and spectrophotometrical determination. As shown in Fig. 4, the activation of caspase 3 started in a linear fashion after 18 h of treatment with 0.6 mM TB. Maximum of caspase 3-activity was measured after 72 h, corresponding to a 5-fold increase relative to control followed by a plateau up to 120 h. Untreated cells exhibited low caspase 3-activity over the incubation time tested.

**Effects of TB on various caspases.** To determine whether the activation of caspases 3, 8 and 9 is central to TB-induced apoptosis, we first exposed LS 174T cells to powerful inhibitors of caspases 3, 8 and 9, respectively. These cells were then treated for 3 days with 0.6 mM TB. Cell lysates...
were assayed for apoptosis inhibition by the nucleosome EIA kit. We found a 6-fold increase of free nucleosomes from 1.4 to 8 U/ml when tumor cells were only incubated with 0.6 mM TB for 3 days (Fig. 5). Compared with TB-treated cells preincubulated with either inhibitors for caspases 3 and 8 apoptosis was prevented. The amount of nucleosomes in the TB-treated cells preincubated with caspase 9 inhibitor was similar to the nucleosome quantities of TB-induced apoptotic cells (9.3 vs. 8 U/ml). Furthermore, no changes in morphology were seen in cultures of TB-treated cells following exposure of caspase inhibitors 3 and 8. Because caspase 9 was not involved in TB-induced cell death we next assessed the secretion of cytosolic levels of cytochrome c which is involved in the mitochondrial pathway of apoptosis. Over a 4-day period with 12-h intervals no changes of cytochrome c release were found (data not shown).

Discussion

Differentiation therapy of cancer is aimed at the induction of differentiation of neoplastic cells and reversal of malignancy. Under normal physiological conditions this process ends in terminal differentiation and subsequent apoptosis (18). The evidence that carcinogenesis involves, to a significant degree, loss or blockade of the normal pattern of cellular differentiation fuels the high priority of this area of cancer research (27,28). The relationship between growth, differentiation and apoptosis of malignant cells seems to be very complex. It is well known that tumor cells, which express a more differentiated phenotype, lose their capacity to proliferate. On the other hand, defective pathways of terminal differentiation lead to autonomous proliferation and prevention of apoptosis (18). It has become apparent that some anticancer drugs can induce apoptosis, for example anthracyclines, cytosine arabinoside and 5-aza-deoxycytidine (30-32). For each of these compounds the dosage has a direct bearing on the mode of cell death. For example, high concentrations of these agents may lead to cell death via necrosis whereas low concentrations are able to induce apoptosis. Actually, less toxic strategies to control cancer progression should be pursued with limited side effects and enhanced survival benefit (33). Furthermore, combinations of different procedures may be more likely to succeed when the agents are chosen to act in a synergistic fashion against multifactorial cancer events (12-15). However, the clinical reality has not provided convincing evidence of the therapeutic benefit of differentiation inducers so far. The maintenance of a constant level of differentiation inducers already at the beginning of tumor progression, where limited defects are evident, seems to be advantageous (29). Animal studies and clinical experience with TB as single agent have shown a 10-fold higher and prolonged plasma level of butyric acid than with sodium butyrate after continuous infusion (8,9). In vitro studies on cellular growth and differentiation demonstrated that TB is 2- to 4-fold more potent than butyric acid on a molar basis (1,3,7). Pharmacokinetic results in humans indicated that frequent application of oral TB is required for effective and sustained plasma butyrate concentrations (8). Hager et al evaluated the clinical toxicity of locally administered TB on patients with advanced cancer. They found that TB is a well-tolerated agent with significant palliative effects and no toxicity even at high doses (10).

Our results demonstrate that treatment of LS 174T colon cancer cells with TB induces dose and time-dependent differentiation and apoptosis, closely linked to the reduction of proliferation, activation of alkaline phosphatase and apoptotic features such as changes in morphology, translocation of phosphatidylserine and activation of selected death proteases. Induction of differentiation measured as ALP activity was linearly enhanced after a 3-day period by concentrations from 0.25 up to 1 mM TB. Apoptotic cells were clearly observed by concentrations higher than 0.6 mM TB. These findings suggest, that both mechanisms are simultaneously acting in LS 174T cells in the concentration range between 0.6 and 1 mM TB.

In agreement with other investigations on MCF-7 cells we have found that TB, after 24 h of exposure, induces a strong decrease (5-fold) of cells in the S-phase and an accumulation (1.3-fold) in the G2/M-phase of cell cycle already at 0.2 mM TB (4). For butyrate on various cancer cells an arrest in the G0/G1-phase was found, reflecting a varying response of different tumor cell lines (4,17). Cellular differentiation as measured by ALP activity was almost linearly increased up to 180-fold with 1 mM TB. Previous studies of TB on proliferation of LS 174T cells have revealed an IC50 value of 0.5 mM. These effects indicated that the differentiation inducing capacity of TB with doses up to 1 mM was inversely related to the loss of proliferation potential. Concentrations higher than 2 mM TB significantly lead to cell death, suggesting that direct cytotoxic effects cannot be excluded in our in vitro system. The TB-mediated apoptosis in LS 174T cells was identified by morphological alterations, externalization of phosphatidylserine and degradation of DNA to generate nucleosomal fragments. Concentrations of TB required to induce apoptosis of LS 174T cells were above the estimated IC50 value. Treatment of cells with 1 mM TB for 3 days caused 50% apoptotic cells using annexin V-FITC labeling whereas ALP activity was maximally induced.

Caspases are important mediators of the initiation steps of apoptosis. The proteolytical activity of caspase 3 of LS 174T cells increased approximately 24 h after exposure of TB followed by a 5-fold induction after a 3-day period. These findings coincided with the appearance of internucleosomal DNA fragments and translocation of phosphatidylserine after 3 days of incubation. Thus, we postulate that caspase 3 plays an essential role in TB-induced apoptosis of LS 174T cells. Furthermore, the involvement of initiator caspases 8 and 9 on TB-induced formation of nucleosomes as indicators for DNA fragmentation was evaluated. We found a 60 and 70% inhibition of apoptosis when tumor cells were treated with 0.6 mM TB and selected peptide inhibitors of caspases 3 and 8, respectively. We have previously shown, that TB increases the expression of the death receptor Fas on LS 174T cells, which may lead to the recruitment of caspase 8 upon ligand binding (15). No changes of apoptotic cells were seen after incubation with a selective caspase 9 inhibitor and subsequent treatment with 0.6 mM TB. Release of cytochrome c from the mitochondrial intermembranous space into the cytosol has been implicated in association with the APAF-1-caspase 9 complex to the proteolytic activation of caspase 3 (34). Our
experiments have shown, that the mitochondrial pathway via cytochrome c release and caspase 9 recruitment is not involved in TB mediated apoptosis of LS 174T cells. Finally, the precise mechanisms mediating caspase activation and cleavage of intracytosolic components are most likely much more complex than currently understood.

In summary, TB may be a promising candidate in sensitizing colon cancer cells to differentiate and to undergo apoptotic death subsequently. The possibility of TB to synergize with other antitumor agents are of interest in clinical oncology to further explore its possible therapeutic potential.

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References