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The effect of the reactive species and the negative energy balance on the development of apoptosis and necrosis

PhD dissertation thesis

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Introduction

The undifferentiated cells of human colorectal adenocarcinoma considered the model cell line for malignant transformation of the colonic epithelial cells. From the parental HT29 line many new cell derivatives were isolated during the prolonged culturing: HT29-12 and HT29-21 are metothrexate and 5-fluorouracil resistant phenotypes. HT29 cell lines can differentiate influenced by different external factors – for example glucose deprivation or butyrate treatment.

In the course of our studies on butyrate-induced proliferation inhibition, detachment and cell death in colon cancer cell lines, we isolated a stable cell line HT29R, which was conspicuous by being virtually refractory to butyrate-induced cell differentiation but highly sensitive to butyrate-induced cell detachment and cell death. We employed HT29R as a model to investigate the pattern of radical and ROS induction by butyrate as related to differentiation, on one side, and cell death, on the other. In several studies it was reported that cell death induction in tumour cell lines by butyrate or other histone deacetylase inhibitors (HDACIs) was associated with enhanced levels of ROS. However, in spite of extensive studies the role of ROS in the basic mechanism, remains to be clarified. Treating cultured cells with millimolar concentrations of butyrate, induces global histone acetylation and manifold effects such as modulation of gene expression, proliferation inhibition, arrest of the cell cycle, induction of differentiation and cell death.

The aim of the present study was to explore the association of various redox parameters with butyrate-induced cell differentiation, detachment and cell death by comparison of the differentiation-resistant but highly cell death-sensitive HT29R cell line with the differentiation-positive and an order of

magnitude less cell death-sensitive HT29-12 and HT29-21 cell lines. To this end, we first examined butyrate-induced proliferation inhibition, differentiation, cell detachment and cell death in HT29R and HT29-21, and subsequently determined the butyrate dose-dependencies of (i) intracellular steady-state free radical levels (FRs) by means of ESR spectrometry, (ii) ROS release as measured by DCF oxidation and (iii) H_2O_2 production as determined with the phenol red assay, (iv) the GSH/GSSG ratio (v) and the redox potential. We wanted to explore whether the differentiation-resistant phenotype of HT29R cells can be characterized by the selective induction or any specific features of butyrate-induced redox parameters. Finally, in HT29R cells, we characterized the mode of cell death induced by butyrate as affected by the presence of 1,5-dihydroxyisoquinoline, resveratrol and cyclsporine A, respectively.

In our further study the physiological decrease of the uterine mass and size after calving, called involution, was studied. In this massive breakdown apoptosis has a central role. Apoptosis makes possible degradation of the endometrium accompanied with cell death without cell membrane disruption and consequent inducing of inflammation.

Dairy cows undergo a period of negative energy balance in puerperium because energy output for milk production exceeds feed energy intake. In high-yielding cows appetite may not be able to satisfy energy requirements. This is exacerbated by the energy demand of the involution, because the more beneficial form of cell death, apoptosis is an energy-requiring process in tissue breakdown. Phosphatidylserine translocation in the membrane, act of the caspase-cascade, DNA fragmentation and forming apoptotic bodies all require energy. Hereafter DNA cleavage activates a repairing process, PARP (PolyADP ribose polimerase) enzyme, which can deplete the ATP of a cell in an attempt to repair the damaged DNA. Finally, PARP can be inactivated by caspase cleavage.

The aim of our second study was to determine the interrelationship between the energy status of the animal and the number of apoptotic cells in the endometrium of uterus biopsy samples collected in the early period of involution. It was supposed that the negative energy balance in the organism causes lack of cellular ATP as well. Consequently, in the negative energy balance the process of apoptosis is disturbed, which increases the risk of necrotic degeneration of epithelial cells and the consequent inflammatory complications.

Materials and methods

In the first experiments the HT29-derived HT29R, HT29-12 and HT29-21 cell lines were cultured in DMEM (Dulbecco's Modified Eagle Medium) at 37°C, at 5% CO_2 content, in a humidified culturing chamber. The cells were treated with various concentration of butyrate for 24 or 48 h then the reaction of the cells was detected.

Proliferation was determined by employing the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) viability assay. Cells were cultured on 96-well plates to determine how the various butyrate concentrations affect on the cell proliferation.

For the detachment experiments, cells were grown in 25 cm^2 flasks. Protein concentration was determined separately from the growth medium with the detached cells and the trypsinized attached cells. Giemsa staining was used for the detection of cell detachment as well. Cells cultivated in 96-well plates were stained for 30 min with a 10% aqueous Giemsa solution.

For the cell death assays method according to Nicoletti was used. The PI-stained nuclei submitted to FACS analysis for determining the cell death related sub-G1 phase. For separating apoptosis and necrosis cells were stained with 2.5 μg/μl propidium iodide and/or 2.5 μg/μl Hoechst 33258 for 10 min. Specimens were examined and photographed by using a fluorescence microscope equipped with an AxioCam digital camera and colour filters.

For TUNEL assay, detecting cells with DNA strand-breaks during apoptotic cell death was used according to the instruction manual. Subsequently, apoptotic cells were selectively labelled by FITC, and then an anti-fluorescein antibody was used to enhance fluorescent intensity. Induction of apoptosis was evaluated by microscopic analysis using a fluorescence microscope and colour filters.

Another apoptosis assay was used according to Quiao et al. by examining the nuclear morphology after staining nuclei with DAPI; necrosis was determined by the trypan blue uptake assay.

Alkaline phosphatase activity was taken as marker for cell differentiation. Alkaline phosphatase activity was measured with p-nitrophenyl phosphate as phosphatase substrate using an ALP kit.

For determining FR and GSH/GSSG concentration cells were grown in 25 cm^2 flasks and treated with sodium butyrate for 48 h. Control and treated cells were frozen and stored in liquid nitrogen for FR and GSH/GSSG determinations. The steady-state free radical concentration was determined using an X-band ESR spectrometer.

To measure reactive oxygen species production, cells were grown in 100 mm tissue culture dishes for 24 h, and thereafter treated with the given butyrate concentrations for 48 h. Then the medium was replaced by dichlorofluorescein (DCF). DCF is converted mainly by ROS to a fluorescent derivate, which can be measured in a spectrophotometer.

The determination of H_2O_2 production was performed according to Baud, so called phenol red method, with adoptive modifications. Cultured cells were incubated with phenol red sodium salt and myeloperoxidase, and then adding base to the solution the absorbance of the complex was read.

Glutathione was assayed using Ellman's-reagent, which reacts with sulfhydryl groups in an alkaline environment and produces a water-soluble complex.

In our second study participated 49 animals of 7 farms in the eastern part of Hungary. The 2-8 years old Holstein Friesian cows were clinically examined on the 4-14 days after calving and according to the results the animals were labelled puerperal metritic or healthy. Blood samples were taken from *Vena jugularis* simultaneously for beta-hydroxybutyrate (BHB) analysis. Cows were

sampled via endometrium biopsy during the early puerperium (on day 4 to 14) to determine the apoptosis index. Biopsy samples were fixed and paraffin embedded.

Paraffin-embedded tissue samples were double immunofluorescence stained for leukocyte detection, common leukocyte antigen (CLA or CD45) and for apoptosis detection, terminal deoxynucleotidyl transferase mediated dUTP nick-end labelling (TUNEL), which can label fragmented DNA. Nuclei were counterstained with fluorescence 4,6-diamino-2-phenylindole (DAPI). Each sample was photographed by using a fluorescence microscope equipped with an AxioCam digital camera and colour filters.

In the photographed sections total endometritic cell number was determined by DAPI-stained cells per field without CLA positive leukocytes. TUNEL positive cells with concurrent DAPI-staining showed the apoptotic cell number. The apoptotic index was calculated by dividing the total number of apoptotic endometrial cells by the total cell number and multiplying by 100.

Results

Alkaline phosphatase activity, which was used as marker of differentiation, was determined in both differentiation-positive cell lines. Induction of alkaline phosphatase activity occurred with a steep dose-dependent increase within the 1-10 mM concentration range of butyrate

As next, we compared the butyrate dose dependencies of proliferation inhibition (MTS viability assay) and cell detachment (Giemsa staining of attached cells) in HT29R and HT29-21 cells, respectively. HT29R cells are highly sensitive to butyrate-induced growth inhibition (as determined with the MTS assay; IC_{50}-value = 1.77 mmol/l) as well as detachment.

Next, we examined the sensitivities too and the mode of cell death induced by butyrate in HT29R and HT29-21 cells. After a 24-h treatment with 10 mM butyrate, 84.6% (control 8.3%) of HT29R nuclear label was detected in the subG1-peak (representative of dead cells), compared with only 8.9% (control 5.0%) of HT29-21 cells. Thus, HT29R cells, resistant to butyrate-induced differentiation, are highly sensitive to butyrate-induced cell death, while the HT29-21 cells are, under the given conditions, virtually resistant to butyrate-induced cell death.

After this, cells were stained with Hoechst 33285 compared with those stained with PI. Hoechst 33285 penetrates through the plasma membrane, and thus stains all nuclei; PI cannot penetrate through the cell membrane, and thus it marks the membrane-damaged late apoptotic and/or necrotic cells. Results show that the higher sensitivity to cell detachment in HT29R is associated with a higher sensitivity to cell death.

As next, we wanted to explore whether resistance to butyrate-induced cell differentiation and/or high sensitivity to cell death are associated with any characteristic features of redox parameters.

There was no significant change in GSH/GSSG ratio and redox potential through the whole range of butyrate concentrations in HT29-12 and HT29-21 cells, while in HT29R, a significant drop at 2-3 mM butyrate occurred.

In HT29R, a marked butyrate dose-dependent increase of the FR levels was induced, whereas in HT29-12 and HT29-21, no FR induction could be detected through the whole range of butyrate concentrations tested.

In HT29R cells, a butyrate dose-dependent induction of ROS/RNOS release was observed, and notably, this did not occur in HT29-12 and HT29-21 cells.

A marked dose-dependent induction of H_2O_2 release, starting at the concentration of 2 mM butyrate, was measured in HT29-12 and HT29-21 cells, whereas no statistically different change of H_2O_2 release could be detected in HT29R through the whole range of butyrate concentrations.

These observations raised the question, whether the butyrate-mediated differential induction of free radicals and/or ROS/RNOS is related to the high sensitivity of cell detachment in HT29R. In HT29-21, cells detachment is induced without concomitant induction of ROS/RNOS or FR production. Showing clearly that, there is no connection between induction of ROS/RNOS or FRs and cell detachment.

Although in HT29R cells, butyrate dose-dependencies of detachment and ROS/RNOS release show a similar pattern, detachment starts at lower concentrations than induction of ROS/RNOS production. However, FRs induction and detachment start at the same butyrate concentrations, a correlation cannot be excluded.

In the following we investigated further the mode of butyrate-induced cell death in HT29R cells. We raised the question how the balance between apoptosis and necrosis is affected by the presence of dihydroquinone (DHQ, an inhibitor of polyADP ribose polymerase), resveratrol (a polyphenolic

phytostilben and antioxidant) or cyclosporine A (CsA, an inhibitor of transition pore opening). Butyrate-induced cell death in HT29R cells is mainly of apoptotic type. In the presence of any of the three agents, namely DHQ, resveratrol or CsA, however, the mode of cell death becomes predominantly apoptotic.

In order to demonstrate the apoptotic mode of cell death by another criterion, we employed the TUNEL technique and anti-PARP staining in examining the effects of DHQ on butyrate-induced cell death. After induction of cell death with butyrate alone, the DAPI-stained blue nuclei appear enlarged, indicative of necrotic cells. In the presence of DHQ, however, a high proportion of cells is apoptotic. Thus, the results of the TUNEL assay confirmed those of microscopical analysis.

In our other *in situ* study before immunostaining one section from each biopsy sample was stained with haematoxylin and eosin for a histological evaluation, and highly infiltrated samples (n = 25) were excluded from the later apoptosis evaluation. According to their uterine status and plasma BHB content, the cows were classified as (i) controls (n = 10), (ii) normoketonaemic-metritic (n = 9), furthermore (iii) hyperketonaemic (BHB: ≥1.00 mmol/l) individuals with or without puerperal metritis (n = 5).

The mean BHB concentration of control animals was 0.45±0.12 mmol/l. In the endometrium biopsy samples 49.97±6.18% of the cells were positive for TUNEL staining apoptosis.

In puerperal metritic, normoketonaemic group the mean of BHB concentration was 0.42±0.15 mmol/l, and the apoptosis index in the endometrium of biopsy samples was 51.67±0.15%.

In hyperketonaemic group the mean of BHB concentration was 1.47±0.32 mmol/l, and the apoptosis index in the endometrium of biopsy samples was 23.83±6.44%.

Comparing the blood BHB concentration data of normo- and hyperketonaemic cows, apoptosis index of the two groups (50.82±4.76% and 23.83±6.44%) indicate significance difference ($p<0.05$).

Discussion

Cell differentiation can be seen as an alternative route to the pathways of continuing proliferation or cell death. This would imply that a checkpoint marks the crossroad between proliferation, differentiation and cell death.

In our study, butyrate dose-dependent induction of H_2O_2 production was detected in the differentiation-sensitive cell lines HT29-12 and HT29-21, and importantly, at the same time neither induction of FRs nor ROS/RNOS release occurred. In contrast to this, in the differentiation-resistant HT29R cells, exactly the opposite was the case, with a butyrate dose-dependent selective induction of FRs and ROS/RNOS without detectable H_2O_2 release. Thus, a selective association of different classes of ROS with either cell-death induction or differentiation was observed, suggesting that they are operative as signalling molecules at the crossroad between differentiation and cell death.

The fact that in HT29R cells the H_2O_2 level remained marginal over the whole range of butyrate concentrations, but in HT29-12 and HT29-21, which are less sensitive to cell death induction by butyrate H_2O_2 release was induced in a dose-dependent manner, suggests that H_2O_2 is not a major player in cell death signalling.

In HT29-12 and HT29-21 cells, at the whole range of the induced H_2O_2 concentrations, the GSH/GSSG ratio and associated redox potential did not change, indicating that this induced H_2O_2, associated with resistance to cell death and sensitivity to differentiation, did not exert oxidative stress.

In HT29R cells, however, the induction of FRs and ROS/RNOS occurring above 1 mM and 5 mM butyrate, was associated with a decrease of cellular GSH/GSSG ratio and change of associated redox potential. Thus, butyrate-mediated induction of FRs and ROS measured in our study represented

oxidative stress in HT29R cells, and was at the same time associated with high sensitivity to cell death.

The mode of cell death induced by butyrate alone only in HT29R cells was mainly of necrotic/late apoptotic type, but in the presence of DHQ, resveratrol or CsA, it was switched to predominantly apoptosis. The enhanced levels of ROS/RNOS under butyrate treatment detected in the present study are consistent with a mechanism of oxidative stress playing an essential role in butyrate-induced cell death. It remains to be elucidated, however, which species of ROS/RNOS are induced by butyrate and what is the underlying mechanism of such induction.

It was experienced in our second study that severe or prolonged deficit of physiologic energy equilibrium results in a disturbed involution and other clinical and subclinical syndromes. The energy-requiring apoptosis is one of the most important elements of involution, consequently programmed cell death is disturbed in the case of negative energy balance. It was concluded that hyperketonaemia related to the postpartum negative energy balance in dairy cows decreases the occurrence of apoptosis in the endometrium. This value was 23.83±6.44% in our experiments.

According to our results in the biopsy samples of the control animals correlating to hyperketonaemic counterparts were less apoptotic cells of the total cell number of the endometrium. In a severe and prolonged negative energy balance apoptosis is perturbed. In case of NEB the cells of endometrium die in a smaller proportion of apopotosis resulting in an increased risk of necrosis and consequent inflammatory processes.

The adequate energy supply of the cows in the puerperium is an important factor for yielding and physiological involution of the uterus as well. If energy requirement satisfies, shrink and restore of endometrium then uterine discharge of tissue debris can be eliminated. The fast and physiological involution is a

guarantee for the uterus not to be a substrate to an overwhelming bacterial reproduction or pathogen contamination that is to the development of puerperal metritis.

New scientific results

In our study we examined *in vitro* in many HT29 cell lines the effect of butyrate, the production of various free radicals and ROS species and the eventual relation to cell proliferation, differentiation, apoptosis and necrosis. We also studied *in situ* in the case of an involved organ, the uterus, the effect of the energetic state of the organism to cell death apoptosis.

I determined:

(1) The butyrate-induced cell differentiation and cell death is in association with the various redox parameters of the HT29 type human colon tumour cell lines.

(2) FRs and ROS arisen by butyrate induction in HT29Rcells are products of cell death, while release of H_2O_2 is generated selectively in HT29-12 and HT29-21 cells in the process of differentiation.

(3) In the early stage of bovine uterine involution the proportion of apoptotic cells was determined in the endometrium of cows with adequate energy supply, as well as of hyperketonaemic cows with negative energy balance. It was established that the number of apoptotic cells decreases by 50% in hyperketonaemic cows compared to that of animals with adequate energy supply.

List of publication

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