Free Rad. Res., Vol. 32, pp. 535–547
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Application of Confocal Laser Scanning Microscopy to Detect Oxidative Stress in Human Colon Cells

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Accepted by Prof. H. Sies

(Received 12 May 1999; In revised form 18 October 1999)

Introduction Excess of intracellular reactive oxygen species in relation to antioxidative systems results in an oxidative environment which may modulate gene expression or damage cellular molecules. These events are expected to greatly contribute to processes of carcinogenesis. Only few studies are available on the oxidative/reductive conditions in the colon, an important tumour target tissue. It was the objective of this work to further develop methods to assess intracellular oxidative stress within human colon cells as a tool to study such associations in nutritional toxicology.

Methods We have measured H_2O_2-induced oxidative stress in different colon cell lines, in freshly isolated human colon crypts, and, for comparative purposes, in NIH3T3 mouse embryo fibroblasts. Detection was performed by loading the cells with the fluorigenic peroxide-sensitive dye 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (diacetoxymethyl ester), followed by *in vitro* treatment with H_2O_2 and fluorescence detection with confocal laser scanning microscopy (CLSM). Using the microgel electrophoresis ("Comet") Assay, we also examined HT29 stem and clone 19A cells and freshly isolated primary colon cells for their relative sensitivity toward H_2O_2-induced DNA damage and for steady-state levels of endogenous oxidative DNA damage.

Results A dose–response relationship was found for the H_2O_2-induced dye decomposition in NIH3T3 cells (7.8–125 μM H_2O_2) whereas no effect occurred in the human colon tumour cell lines HT29 stem and HT29 clone 19A (62–1000 μM H_2O_2). Fluorescence was significantly increased at 62 μM H_2O_2 in the human colon adenocarcinoma cell line Caco-2. In isolated human colon crypts, the lower crypt cells (targets of colon cancer) were more sensitive towards H_2O_2 than the more differentiated upper crypt cells. In contrast to the CLSM results, oxidative DNA damage was detected in both cell lines using the Comet Assay. Endogenous oxidative DNA damage was highest in HT29 clone 19A, followed by the primary colon cells and HT29 stem cells.

Conclusions Oxidative stress in colon cells leads to damage of macromolecules which is sensitively detected in the Comet Assay. The lacking response of the CLSM-approach in colon tumour cells is probably due to intrinsic modes of protective activities of these cells. In general, however, the CLSM method is a sensitive technique to detect very low concentrations

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of H_2O_2-induced oxidative stress in NIH3T3 cells. Moreover, by using colon crypts it provides the unique possibility of assessing cell specific levels of oxidative stress in explanted human tissues. Our results demonstrate that the actual target cells of colon cancer induction are indeed susceptible to the oxidative activity of H_2O_2.

Keywords: Confocal laser scanning microscope, oxidative stress, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, human colon cells, human colon crypts, Comet Assay, endonuclease III

INTRODUCTION

Mammalian cells may be exposed to reactive oxygen species (ROS) arising from a manifold of different sources. These include diverse oxygen radicals and products of peroxides formed during energy metabolism, transformation of toxic agents and lipid peroxidation.[1] Also, it has been proposed that H_2O_2 is formed as a second messenger during signal transduction induced by growth factor activation.[2] The action of ROS is counteracted by enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione S-transferases or by scavenging molecules, such as glutathione and other peptides.[3] Generally, it is accepted that an excess of ROS in relation to antioxidative systems will result in oxidative stress which will cause oxidative damage to DNA, proteins or lipids.[4,5] Alternatively, recent observations indicate that modulation of oxidative/reductive intracellular conditions may be strong determinants in regulating gene expression.[6] This postulation is supported by findings indicating that cystein residues of zinc finger motives found in transcription factors must be in a reduced state for effective DNA binding. They are redox-sensitive and accordingly, the corresponding gene activation is dependent on the redox environment within the cell.[7] Thus, it is important to determine oxidative conditions within living cells, for which new techniques, enabling ROS detection are required. A basis for these techniques is the availability of useful markers for ROS detection. One of these is dichlorofluorescein diacetate which has been used to determine *tert*-butyl hydroperoxide-induced oxidative stress in NIH3T3 cells and derivatives thereof.[8] The analysis is based on the permeability of the esterified compound, which freely passes into the cell and there is cleaved by esterases into a species which is effectively retained within the cell. It decomposes to a fluorescent analogue in oxidative conditions.

We have now used a further developed derivative of dichlorodifluorescein (DCF), the 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (diacetoxymethyl ester), which should more easily pass through the cell membrane during cell loading and is more efficiently retained in the cell after cleavage.[9] With this compound, we have compared the sensitivity towards hydrogen peroxide of NIH3T3 cells, of three different human colon tumour cell lines, and of primary colon cells within intact crypts from human biopsies. The aim of this work is to assess toxicological parameters (in this case: oxidative stress) within specific, individual living cells in order to elucidate etiological risk factors of colon carcinogenesis, especially those which may act as oxidants.[10,11] These techniques in the future may enable us to detect the modulation of these parameters by antioxidants/anticarcinogens from the diet.[12,13]

In this first study, we have now investigated different concentrations of H_2O_2 in human colon tumour cell lines resembling adult, differentiated cells (HT29 clone 19A) and have compared the effects to those obtained with cells more resembling embryonic, undifferentiated cells (HT29 stem, Caco-2).[14,15] The mouse embryo fibroblast cell line (NIH3T3) was included as it had been reported to be a sensitive target of DCF oxidation.[8] Top (differentiated) and bottom (undifferentiated) regions of intact colon crypts were compared for their sensitivity against oxidative stress, as well.

In a further set of experiments, we examined endogenous and induced DNA damage of HT29

stem, HT29 clone 19A and primary human colon cells using the Comet Assay.[16] Migration of damaged DNA in the electric field gives rise to comet-like structures.[17] The fluorescence in tail of nuclei from untreated cells reflects the amount of endogenous DNA breaks, alkali labile sites, apurinic and apyrimidinic sites, and single stranded DNA, resulting from repair or replication and the utilization of repair specific enzymes reveals oxidized DNA bases.[18]

MATERIALS AND METHODS

Cell Culture

Human Tumour Cell Lines HT29 Stem and HT29 Clone 19A

The human colon cell line HT29 was established by J. Fogh (Memorial Sloan Kettering Cancer Centre, New York) in 1964. It was obtained from the American Tissue Culture Collection (ATCC), Rockville, MD, USA. The subclone 19A was terminally differentiated with 5 mM sodium butyrate, characterized by Augeron and Laboisse and was a kind gift of Laboisse.[15] Cells were maintained in stocks in liquid nitrogen. All culture media and components were from Life Technologies (Eggenstein, Germany). Cells were thawed and grown in tissue culture flasks with DMEM supplemented with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin at 37°C in a humidified incubator (5% CO_2/95% air). This culture containing 45–60 $\times 10^6$ cells was trypsinized using 1–1.5 ml trypsin/versene (1 : 10 v/v) for a maximum of 10 min and subcultivated at a dilution of 1 : 8 in T_{75} flasks with supplemented DMEM. Two medium changes occurred on days two and five with 16 ml supplemented DMEM. For the Comet Assay, the cells were trypsinized and resuspended in phosphate buffered saline (PBS). Cell number and viability were determined using trypan blue. The cell number was adjusted to 2×10^6 cells/ml.

Caco-2

The human colon adenocarcinoma cell line Caco-2 was obtained from ATCC, Rockville, MD, USA. Cells were grown in DMEM (with 4.5 g glucose/l and 25 mM HEPES), supplemented with 10% (v/v) FCS, 2 mM glutamine, 0.11 g/l Na-pyruvate, 0.5% (v/v) penicillin/streptomycin and 1% (v/v) non-essential amino acids (100× solution). To subcultivate the cells, they were diluted 1 : 8 after trypsinizing. Medium changes were on days two and five.

NIH3T3

The mouse embryo fibroblast cell line was obtained from the tissue culture collection at the German Cancer Research Centre (Heidelberg, Germany). NIH3T3 cells were cultivated in RPMI 1640 including 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin and 2 mM glutamine. Dilution after trypsinization was 1 : 10, medium change was carried out every 2–3 days.

Cultivation of Cells on Cover Slips

For the laser scanning microscopy (LSM) experiments, cells were seeded in 3 ml medium in culture dishes (55 mm diameter) containing a sterile cover slip (33 mm diameter) and cultivated for 3–5 days until they had reached the optimal cell density (70–80% confluence). Medium was changed 24 h before the experiment. To load the cells with carbDCF, the cover slips were removed from the culture dishes and fixed in special perfusion chambers, which fitted to the microscope table.

Donors of Colon Biopsies

The biopsy donors were admitted to the hospitals for various reasons and were subjected to diagnostic colonoscopy to exclude either polyps or colorectal carcinoma after occult faecal blood test had been positive. Mean age (±SD) of all donors

was 63 ± 13.8 years ($n = 33$), 67% of the donors were male, 33% female. The excision of biopsies was performed after the patients gave their informed consent. In all patients the findings were negative with respect to benign or malignant tumours or inflammatory bowel disease. Six biopsies were obtained from macroscopically healthy colon sigmoid tissue, stored in Hank's balanced salt solution and transported on ice to the laboratory within 1–2 h. The study was approved by an ethics committee (Landesärztekammer Baden-Württemberg).

Isolation of Primary Human Colon Cells

Primary colon cells were obtained by incubating and mincing the colon biopsies in 3 ml Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} and supplemented with 6 mg proteinase K (Amresco, Solon, Ohio, USA) and 3 mg collagenase P (Boehringer Mannheim, Germany) for 30 min in a shaking water bath at 37°C. The suspensions were then diluted with HBSS to a final volume of 15 ml and centrifuged for 6 min at $139 \times g$. The pellet, consisting of colonocytes, was resuspended in 1 ml PBS. Viability and cell yield were determined in a haemocytometer using trypan blue and the cell number was adjusted to 2×10^6 cells/ml.

Isolation of Primary Human Colon Crypts for Confocal LSM (CLSM)

The biopsy samples were minced with fine scissors and incubated in 3 ml HBSS (without Ca^{2+} and Mg^{2+}) supplemented with 6 mg proteinase K and 3 mg collagenase P for 15 min in a shaking water bath at 37°C. The suspensions were then diluted with HBSS to a final volume of 15 ml and centrifuged for 6 min at $139 \times g$. Pellets containing isolated colon crypts but also numerous single cells were gently resuspended in 2 ml HBSS (including Ca^{2+} and Mg^{2+}). One ml of this solution was loaded onto a cover slip which was fixed in a perfusion chamber and which had been pre-treated with 10 µl of a cell and tissue adhesive (Cell-TAK, Collaborative Biomedical Products, Bedford, MA, USA). The crypts were allowed to attach to the cover slip for a minimum of 15 min on ice, then they were immediately used for the detection of oxidative stress.

Incubation with CarbDCF

6-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di-acetoxymethyl ester, (CarbDCF, Molecular Probes, Leiden, Netherlands) is a non-fluorescent, oxidant-sensitive dye. Modification of carboxylic acids with acetoxymethyl ester groups results in an uncharged molecule that can penetrate the cell membranes. Once inside the cell, these lipophilic groups are cleaved by non-specific esterases, resulting in a charged free acid form that leaks out of the cell far more slowly than its parent compound. Upon oxidation, carbDCF becomes fluorescent.

CarbDCF was dissolved in DMSO and stored as a 5 mM stock solution at −20°C. A 10 µM working solution was prepared in HBSS (including Ca^{2+} and Mg^{2+}), supplemented with 5 mg BSA/ml freshly before use.

The cells or colon crypts attached on the cover slips and fixed in the perfusion chamber were loaded with 1.5 ml carbDCF working solution and incubated at 30°C for 15 min with moderate shaking. The carbDCF solution was then replaced by fresh HBSS (including Ca^{2+} and Mg^{2+}), the chamber was fixed on a thermostatized chamber on the microscope table of the CLSM and perfused at 37°C with HBSS (1.5 ml/min) using a peristaltic pump (Abimed Gilson, Villiers-le-Bel, France). The colon crypts selected for the experiments had retained their integrity and showed no morphological signs of toxicity at the beginning of the HBSS perfusion.

Confocal Laser Scanning Microscopy

We used a Zeiss Confocal Laser Scan Microscope (LSM 410 invert, Carl Zeiss, Jena, Germany).

The Ar 488 nm and HeNe 543 nm laser lines were used for fluorescence detection and transmission microscopy, respectively. For the Ar 488 nm laser, an attenuation of 1 : 1000 was used. Fluorescence and transmitted light were detected after passage through a 515 nm long pass emission filter. The magnification of the objective was 40× with a numeric aperture of 1.2. Colon crypts were examined using the confocal mode with a full width half maximum (FWHM) of 2.9 μm. The cell lines were measured non-confocally. All experiments were performed under red safety light.

We first defined six regions of interest (ROI), each corresponding to a single cell, or to nearly a single cell in the case of the colon crypts, and one region of interest, defining the background of the cover slip. For the colon crypts, ROIs 1–3 were placed near the lower part of the crypt, ROIs 4–6 near the top of the crypt to study differences in sensitivity between these two regions. The chambers were perfused at 37°C for 300 s with HBSS, followed by different concentrations of H_2O_2 (in HBSS) for another 600 s. The controls received HBSS for the whole time period. The intensity of the fluorescent dye in all defined ROIs was monitored over time (one image every 10 s).

Transmission and fluorescence images of the cells were stored before and after the H_2O_2-treatment (time points 0 and 900 s).

For better comparison of the diverse experiments, the background value from a cell free area was subtracted. Relative changes were calculated by setting the initial fluorescence at zero time (measured as absolute grey levels) = 100%. An absolute increase in fluorescence from 10 to 100 (grey levels) at the end of the perfusion ($t = 900$ s) would result in a relative increase of 1000%.

Statistical Evaluation (CLSM)

Each determination was independently reproduced several times. Between three and eight individual determinations are available for each data point, expressed as relative intensity (% of zero time). The effect of H_2O_2-treatment is analysed by comparing time point 900 s of treated and untreated samples using a two-sided, unpaired *t*-test. For the colon crypts, a two-sided, paired *t*-test was applied to compare the effects of H_2O_2-treatment in upper versus lower crypt cells of the same colon crypt.

Genetic Damage with the Microgel-Electrophoresis ("Comet") Assay

To determine H_2O_2-induced DNA damage, 2×10^5 cells (100 μl of the adjusted cell suspension) were distributed with 75 μl 0.7% (w/v) low melting agarose (in PBS) on microscopical slides. After 10 min the layer was covered with another layer of agarose (75 μl, 0.7% (w/v) low melting agarose). The slides were placed on ice cold racks, treated with 50 μl physiological NaCl or with 50 μl H_2O_2 (100 μM) for 5 min and then placed into lysis solution for at least 1 h. Subsequently, the Comet Assay was performed as described previously.[19] To determine oxidized DNA bases, we used our protocol of the Comet Assay and additionally the modification with endonuclease III (kind gift of Dr. Collins, UK) to detect levels of oxidized pyrimidine bases.[16]

Evaluation of the images on the slides was performed by microscopical analysis. Using the imaging software of Perceptive Instruments (Halstead, UK), 50 images were evaluated per slide and the percentage of fluorescence in the tail (= TI, "tail intensity") was scored.

Statistical Evaluation (Comet Assay)

A Welch-corrected two-tailed, unpaired *t*-test was applied to compare individual slides of H_2O_2-treated cells and of endonuclease III treated slides to their respective controls. Each experiment was repeated independently several times. Means of three slides per experiment (experiments with cultured cells) and of one slide per experiment (primary colon cells) were taken as basis for calculating mean ± SEM for each data point.

RESULTS

CarbDCF effectively decomposes to yield a fluorescent intermediate upon oxidative stress in NIH3T3 cells. These mouse embryo fibroblasts only weakly react towards endogenous stress occurring within the 900 s of incubation, as can be seen in Figure 1. H_2O_2 is increasingly effective beginning at 7.8 μM up to 125 μM, where a relative intensity of 900 is achieved. Figure 2 shows an example of NIH3T3 cells before (A and B) and after (C and D) treatment with 125 μM H_2O_2. At higher concentrations, lower effects are observed probably due to toxicity. This may lead to inactivation of peroxidases and thus less liberation of oxidizing intermediates. Also, the toxicity could affect the cell membrane and result in leakage of the fluorescent compound from the cell. Schwarz *et al.*[8] have used this approach to compare the relative sensitivities of NIH3T3 cells with different levels of metallothionein expression. With 500 μM *tert*-butyl hydroperoxide as the oxidising agent and DCF as the redox-sensitive fluorigenic compound, a 1000% increase was observed after 60 s in the wild type NIH3T3 strain also used here. They observed similar diffuse increases of fluorescence in all exposed cells for DCF as we did for carbDCF.

In non-differentiated colon tumour cells (HT29 stem cells), endogenous oxidative stress as a result of incubating the cells *in vitro* with H_2O_2 did not increase the carbDCF fluorescent-conversion. Figure 3(a) shows the results at 62, 250 and 1000 μM H_2O_2. This may be due to a decreased sensitivity of the cells to these environmental conditions. Interestingly, the more differentiated cell line HT29 clone 19A is also not sensitive towards H_2O_2. As is shown in Figure 3(b), no statistical significant activity is observed up to 1000 μM. For untreated Caco-2 cells (Figure 3(c)), we found a slight reduction of the fluorescence during the 900 s, whereas they tend to be more sensitive to H_2O_2 action than HT29 stem and clone 19A cells: 62.5 μM

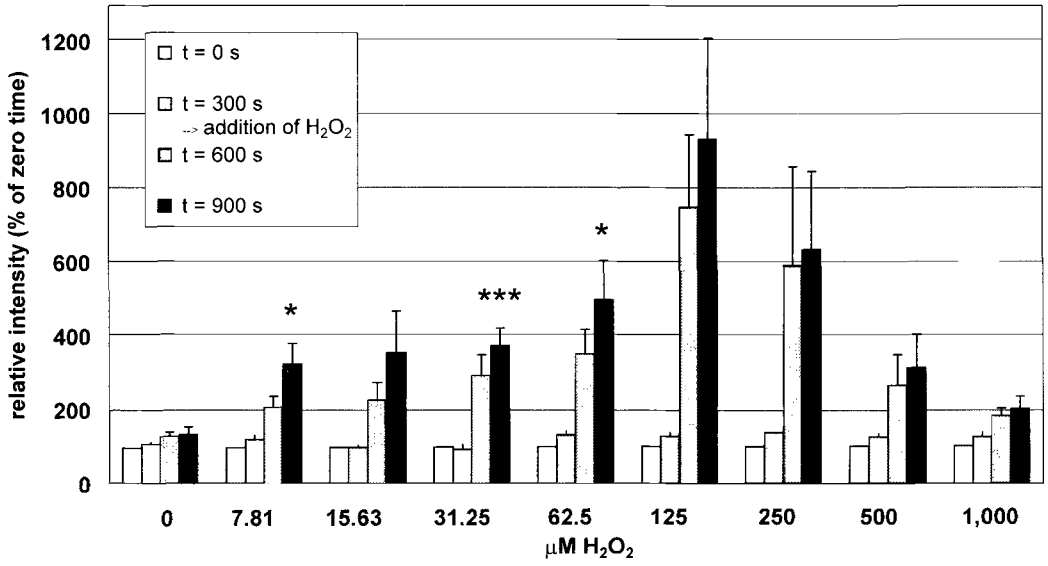


FIGURE 1 Oxidation of carbDCF to its fluorigenic product in NIH3T3 cells. Changes in the fluorescence intensity over time (value at time point 0 = 100%). Cells were perfused with HBSS from 0–300 s and with different concentrations (7.8–1000 μM) of H_2O_2 from 300–900 s. Controls were perfused with HBSS for the whole 900 s. Means of 3–8 independent experiments (±SEM) are shown for each concentration. A two-tailed, unpaired *t*-test was applied to compare the relative intensity following H_2O_2-treatment to the intensity of control cells ($t = 900$ s, $^*p < 0.05$; $^{***}p < 0.0001$).

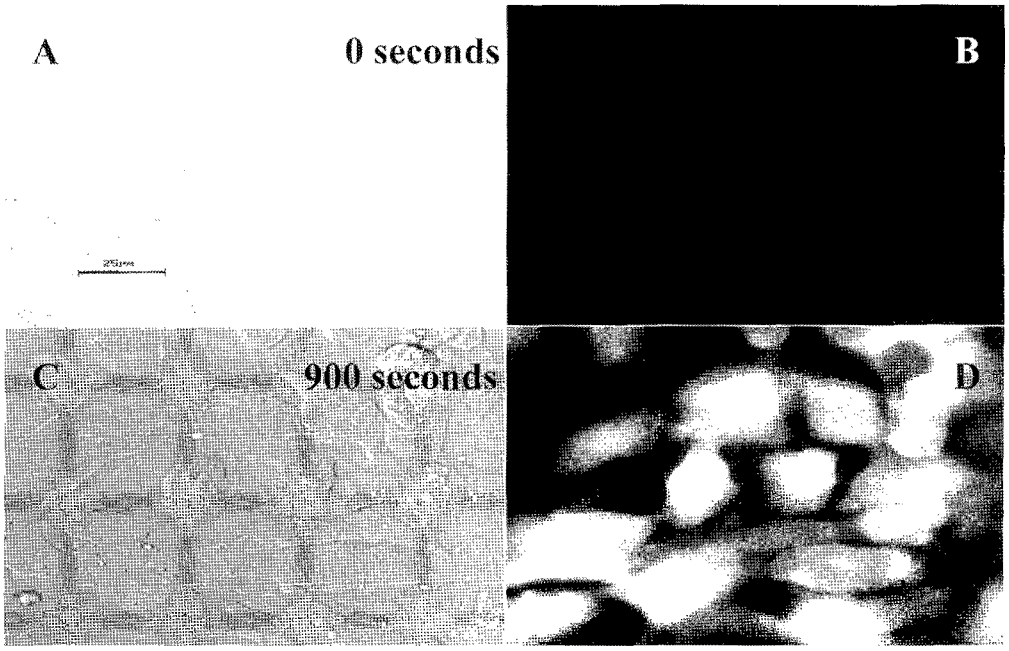


FIGURE 2 Effect of 125 μM H_2O_2 on carbDCF fluorescence in NIH3T3 cells. Upper panels show the transmission picture (A) and the fluorescence picture (B) before H_2O_2-perfusion (0 s). Lower panels (C, D) show the same cells after H_2O_2-treatment (900 s). Fluorescence has strongly increased due to oxidative stress. The scale bar indicates 25 μM.

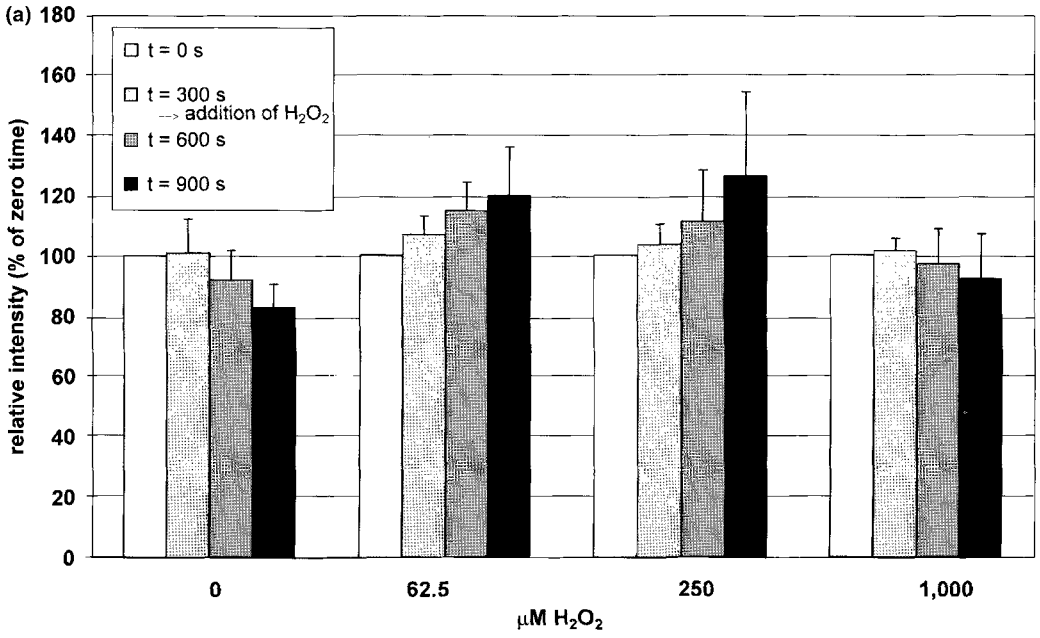
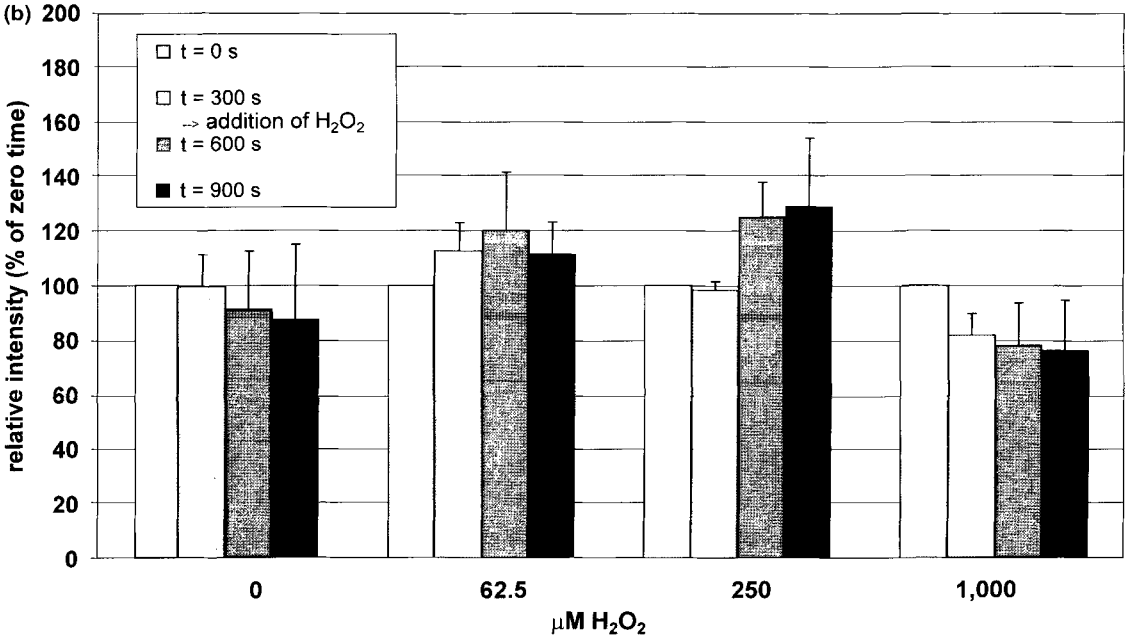


FIGURE 3(a)



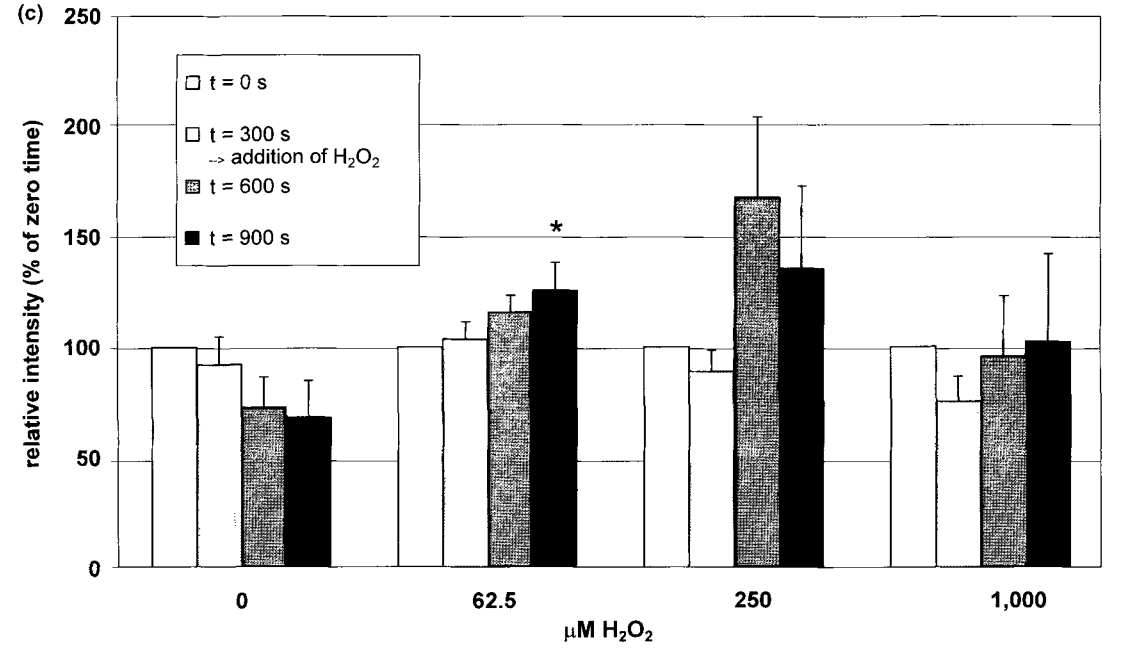


FIGURE 3(b) and (c)

FIGURE 3 Oxidation of carbDCF to its fluorigenic product in three different human colon tumour cell lines (a: HT29 stem; b: HT29 clone 19A; c: Caco-2). Changes in the fluorescent intensity over time (value at time point 0 = 100%). Cells were perfused with HBSS from 0–300 s and, with different concentrations (62.5–1000 µM) of H_2O_2 from 300–900 s. Controls were perfused with HBSS for the whole 900 s. Means of 3–6 independent experiments (±SEM) are shown for each concentration. A two-tailed, unpaired *t*-test was applied to compare the relative intensity following H_2O_2-treatment to the intensity of control cells ($t = 900$ s). *$p < 0.05$.

H_2O_2 significantly increased the fluorescence intensity.

In order to more closely determine the actual situation of oxidative stress in the non-transformed human colon, it is necessary to investigate the actual target cells and not surrogates (colon tumour cell lines etc.) thereof. Therefore, we have started to develop the technique further and to apply it to cells from intact colon crypts.

Crypts were isolated from colon biopsies and the sensitivity of lower crypt cells (ROIs 1–3) was compared to upper crypt cells (ROIs 4–6) of the same crypt. An example of a human colon crypt before (A and B) and after (C and D) H_2O_2 perfusion is shown in Figure 4. Figure 5 shows that there is no difference during incubation with the solvent alone. At 125 μM H_2O_2, however, cells 1–3 are more sensitive than cells 4–6, a trend which is also present at 250 μM H_2O_2 and continues to be apparent at 500 and 1000 μM H_2O_2 (Figure 5). The differences at 500 and 1000 μM H_2O_2, however, are not statistically significant.

The results of the Comet Assay are shown in Figure 6. One hundred μM H_2O_2 as well as 45 min endonuclease III treatment significantly increased the tail intensity in the two colon tumour cell lines and in the primary colonocytes ($p < 0.0001$). It is, however, obvious that there are differences in the sensitivity of the different cells towards H_2O_2 and differences in steady-state levels of oxidized pyrimidine bases as determined with endonuclease III. In both cases, the more differentiated cell line HT29 clone 19A exhibited the highest increase in tail intensity. The extent of H_2O_2-induced DNA damage was lowest in the primary colon cells followed by HT29 stem cells, whereas for endogenous oxidized DNA bases, HT29 stem cells showed the lowest increase of the three different cell types.

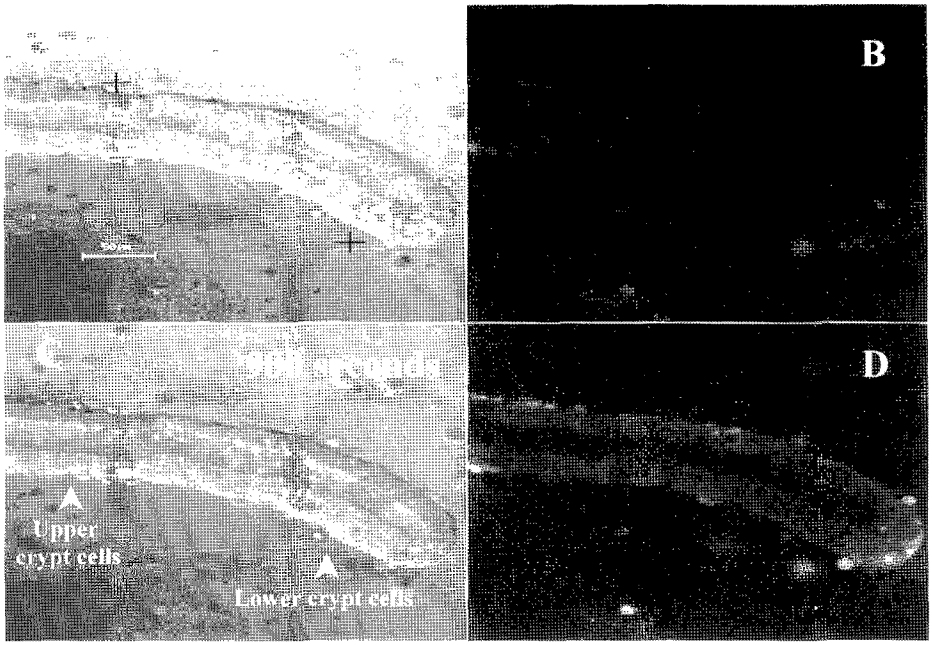


FIGURE 4 Effect of 1000 μM H_2O_2 on carbDCF fluorescence in a freshly isolated human colon crypt. Upper panels show the transmission picture (A) and the fluorescence picture (B) before H_2O_2-perfusion (0 s). Lower panels show the same crypt after H_2O_2-treatment (900 s). The carbDCF fluorescence (D) has markedly increased in the crypt cells. The colon crypt (C) has retained its integrity and shape during the whole experiment. The scale bar indicates 50 μM.

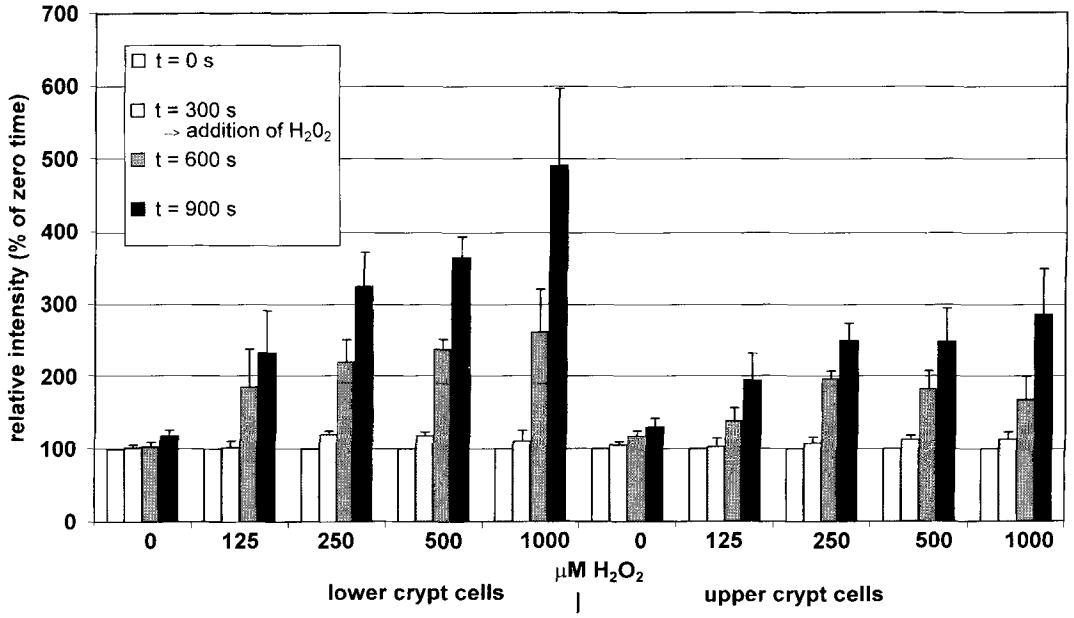


FIGURE 5 Oxidation of carbDCF to its fluorigenic product in upper and lower areas of primary human colon crypts. Changes in the fluorescent intensity over time (time point 0 = 100%). Colon crypts were perfused with HBSS from 0–300 s and with different concentrations (125–1000 µM) of H_2O_2 from 300–900 s. Controls were perfused with HBSS for the whole 900 s. Means of 5–7 independent experiments (±SEM) are shown for each concentration. A two-tailed, paired *t*-test was applied to compare the relative intensities of upper and lower crypt cells (same crypts) following HBSS or H_2O_2-treatment at $t = 900$ s.

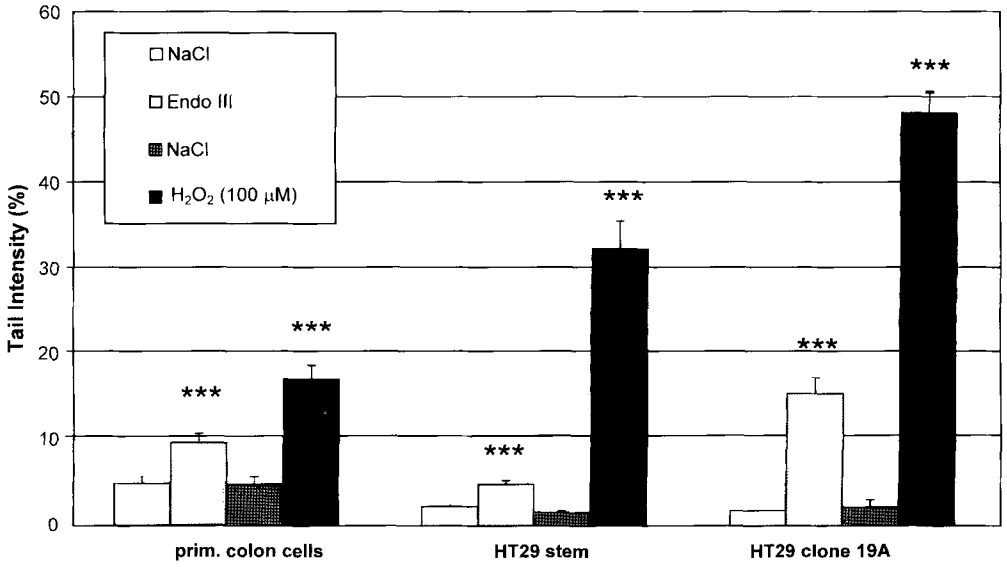


FIGURE 6 Determination of DNA damage using the Comet Assay. Extent of DNA damage (expressed as % fluorescence in the tail) in primary human colonocytes, HT29 stem and clone 19A cells following 5 min 100 µM H_2O_2 or 45 min endonuclease III incubation (mean ± SEM; $n \geq 21$). A two-tailed, unpaired *t*-test was applied to compare the treatment groups with their NaCl controls (***$p < 0.0001$).

DISCUSSION

We have measured H_2O_2-induced oxidative stress in different colon cell lines, in freshly isolated human colon crypts, and in NIH3T3 mouse embryo fibroblasts using the oxidant-sensitive compound carbDCF. NIH3T3 cells as well as intact colon crypts revealed a dose–response relationship with increasing H_2O_2-concentrations. When comparing those crypt cells located in the bottom region to the upper crypt cells, the cells in the bottom region were more susceptible to H_2O_2.

The use of isolated crypts from human colon biopsies has four major advantages: (1) relevant human target cells of colon cancer can be analysed, (2) the cells are non-transformed and can be used for studies in cancer prevention, (3) it is possible to discriminate effects in differentiated colon cells of the upper crypt region and the bottom of the crypt region, (4) concomitant toxic effects can be observed as "blebbing".

According to our knowledge, this is the first demonstration of oxidative stress within intact living primary colon cells (non-transformed). Also, it is the first time that human crypts have been used in a study that combines the use of carbDCF and CLSM. So far, only one group has reported an investigation with rat crypts, which, however, were not living but were fixed prior to microscopic investigation.[20] Our studies show that the actual target cells of cancer induction are susceptible to the oxidation of H_2O_2. These cells are located in the bottom regions of the crypts where the stem cells, from which tumours actually are expected to arise, are located. Similar observations are described by Brooks and Winton[21] who used a modification of the Comet Assay to detect DNA damage in rat intestinal crypts. They found larger comet tails in the lower crypt cells when exposing the crypts to 6.8 μM H_2O_2 for 30 min. At higher doses, however, all regions of the crypt appeared to contribute to the comet.[21]

The sensitivity of the primary colon cells is intrinsically different from the sensitivity of tumour cell lines which show no activity under the same experimental conditions. Comparatively less oxidative stress is detectable in the primary human colon cells than in mouse embryo derived NIH3T3 cells. Additional experiments are needed to check if the different sensitivities of primary and tumour cells as well as of upper and lower crypt cells are due to different loading capacities of carbDCF. This could possibly be achieved by loading the cells with a different dye such as fluorescein diacetate to compare the fluorescence intensities.

Although we did not detect H_2O_2-induced oxidative stress in transformed human HT29 cells (stem and clone 19A), there was a strong increase in DNA damage following H_2O_2-incubation as measured in the Comet Assay. This could indicate the presence of intrinsic protection against ROS in the cytosol of these cells. This hypothesis is strengthened by findings of Schwarz *et al.*,[8] who showed that NIH3T3 cells that overexpress metallothionein have an increased antioxidant activity, but this metallothionein overexpression did not protect the cells from *tert*-butyl hydroperoxide-induced DNA damage. Endogenous oxidative DNA damage as measured using the endonuclease III modification revealed that the undifferentiated cell line HT29 stem has the lowest level of endogenous damage, followed by the primary colon cells and the more differentiated subclone 19A. Alternatively, the cells are not insensitive towards oxidative stress, instead there may be a specific mechanism yielding negative results during the CLSM-measurements. Theoretically, extensive extrusion of the dye carbDCF may be responsible for the lacking detection of oxidative stress in these cells. Fluorescent organic anions and cations can be extruded from cancer cells through specific transport systems like the multidrug resistance transporter, P-glycoprotein, and the multidrug resistance-associated protein (MRP).[22] In fact, with respect to carbDCF a lower accumulation of the fluorescent dyes carboxy-2′,7′-dichlorofluorescein and 2′,7′-bis-(carboxyethyl)-5(6)-carboxyfluorescein and

2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein has been observed in MRP-overexpressing cells.[23,24] However, this explanation seems unlikely, since both Caco-2 cells and intestinal epithelium cells are known to express such transport systems[25] and, at least in our experiments, retained sufficient intracellular carbDCF to sensitively detect oxidative stress by LSM and CLSM, respectively. In summary, regarding the results with intact colon crypts, this technology holds great potential for the study of etiological risk factors and protective agents in human colon carcinogenesis. Specifically, it will be more possible to study the role of individual nutritional factors and their relative balance on inducing and preventing cellular stress (oxidative and genotoxic) within tumour target cells.

Acknowledgements

We thank Prof. Gugler and Dr. Kark, Städtisches Klinikum Karlsruhe, as well as Prof. Siegel, Vincentius-Krankenhaus Karlsruhe, for providing us with the human colon biopsies. The technical assistance of Mrs. Renate Lambertz, Mr. Martin Knoll, Mrs. Susanne Merkel and Mrs. Cornelia Stoltz is greatly acknowledged. This work was supported by the Deutsche Krebshilfe Project No. 70-2165-Po2, and by the European Community, Project No. FAIR-CT-95-0894.

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