APOPTOTIC PROCESS IN THE MONKEY SMALL INTESTINAL EPITHELIUM: 2. POSSIBLE ROLE OF OXIDATIVE STRESS

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Abstract—Recent findings suggest that intracellular oxidants are involved in the induction of apoptosis and this type of cell death can be inhibited by various antioxidants. In our accompanying paper, we have shown apoptosis in the villus tip cells of the monkey small intestinal epithelium. The aim of the present study was to evaluate the possible relationship between oxidative stress, antioxidant levels and the apoptotic process in the monkey small intestinal epithelium. Monkey small intestinal epithelial cells were isolated into different fractions consisting of villus, middle and crypt cells. Mitochondrial function was assessed by the reduction of the tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), with and without succinate. The extent of lipid peroxidation was assessed by measuring the formation of conjugated diene, depletion of polyunsaturated fatty acids and \( \alpha \)-tocopherol. Level of antioxidant enzymes like, superoxide dismutase (SOD), catalase, glutathione S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase were also quantitated in various cell fractions. MTT reduction was significantly decreased in villus cells as compared to the cells from other fractions and this was evident even in presence of the respiratory substrate, succinate. Increased formation of conjugated diene and depletion of polyunsaturated fatty acids were seen in villus and crypt cells as compared to middle fraction cells. The \( \alpha \)-tocopherol level was decreased in both villus and crypt cells as compared to cells from middle region. Significant decrease of SOD activity was seen in the villus tip cells and a slight decrease was seen in the crypt fractions. Glutathione dependent enzymes like GST, GPx and GSH reductase showed higher activity in the villus fractions. A similar observation was also seen in the catalase activity. This study has shown that although oxidative stress is seen in both villus and crypt cells, decreased mitochondrial function was seen in villus tip cells which may be responsible for apoptotic process in the intestinal epithelium. © 1998 Elsevier Science Inc.

Keywords—Apoptosis, Monkey, Intestinal epithelium, Oxidative stress, Free Radicals

INTRODUCTION

Aerobic organisms faced with the threat of oxidation from molecular oxygen (O\(_2\)) [1] have evolved antioxidant defenses to cope with this potential problem. However, cellular antioxidants can become overwhelmed by oxidative insults, including supraphysiologic concentration of O\(_2\). Oxidative cell injury involves modifications of cellular macromolecules by reactive oxygen intermediates (ROI), often leading to cell death [2,3]. There is ample evidence that apoptosis is accompanied by oxidative stress [4]. It has recently become apparent that ROI can have divergent effects on mammalian cell growth. In some cases, low doses of oxygen free radicals induce cells to undergo apoptosis, or programmed cell death [5], whereas in other cases reactive oxygen species were found to promote cell proliferation [6,7]. Such findings suggest that ROI may function as intracellular second messengers. Evidences suggest that oxidative stress may be a common mediator of apoptosis, perhaps via the formation of lipid hydroperoxides [4,8,9,]. Indeed, lipid hydroperoxides are known to be toxic [9,10], to increase cytosolic Ca\(^{2+}\) [11], and to promote DNA fragmentation [9,12]. Recent work has shown that oxidants can induce apoptosis [4] and that Bcl-2, a constitutive protein inhibitor of apoptosis, may have a role as an antioxidant, or as a modulator of the oxidant status of the cell [13,14]. The level of Bcl-2 protein was higher in proliferating cells, whereas in differentiated cells, this protein was replaced
by an enhanced expression of glutathione-S-transferase. These substitutions display an adaptive response of the cell and may be linked to the redox potential, which is a critical determinant in cell proliferation, differentiation and apoptosis [15]. Oxidative stress can be induced by decreasing the ability of a cell to scavenge or detoxify ROI. For example, compounds such as buthionine sulfoximine (BSO) deplete intracellular stores of glutathione (GSH), thereby rendering cells more susceptible to oxidative stress-induced apoptosis [16,17]. We have demonstrated apoptosis in the villus tip cells of monkey small intestine and this is associated with the efflux of GSH (accompanying paper).

Within the mucosal environment of the gastrointestinal tract, multiple cytokine and cell-mediated signals can lead to epithelial cell injury. One form of epithelial cell injury is apoptosis [18,19]. Although apoptosis is part of the normal process of epithelial cell renewal [20], in excess it is pathologic. An overwhelming number of apoptosis-inducing stimuli have been described in various cell systems. In certain systems, the continuous presence of growth or survival factors is required for the inhibition of apoptosis [21,22].

The enterocytes along the crypt-villus axis in the small intestine have specialized function and structure. The villus tip enterocytes are well differentiated and possess various proteins, which are involved in digestion and absorption [23]. Earlier reports suggested that apoptosis was responsible for shedding of cells from the villus tips in the small intestine [24]. In contrast, Merritt et al. has identified apoptosis only in the crypts of the murine small intestine and colon [25]. During apoptosis, cells shrink, microvilli disappear, and the cell may detach from neighboring cells. The apoptosis is often unclear and difficult to detect in vivo [26]. The intestinal epithelium is an ideal system to study the apoptotic process under in vivo conditions, due to rapid turnover of cells.

In this report we have looked at the possible role of oxidative stress and antioxidant protection in the apoptotic process of the monkey small intestinal epithelium.

**MATERIALS AND METHODS**

Bovine serum albumin, succinate, MTT, dimethyl sulfoxide (DMSO), superoxide dismutase (SOD), NADPH, glutathione (GSH, GSSG), catalase, glutathione reductase, dithiothreitol (DTT), and EDTA, were all obtained from Sigma Chemical Co. USA. All other chemicals used were of analytical grade. Microtiter plates were supplied by Sterlin, Middlesex (England), 1-chloro-2,4-dinitrobenzene (CDNB), was obtained from BDH (London). Sodium azide (NaN3) was obtained from Fluka Chemie AG, Switzerland.

**Isolation of enterocytes**

Enterocytes were isolated from the monkey small intestine as described earlier [27] and purity judged by assay of alkaline phosphatase activity [28].

**Measurement of cell viability**

Viability of isolated cells was assessed by trypan blue exclusion and lactate dehydrogenase release in the medium, using kinetic assay [29]. Cell homogenates were prepared in phosphate buffered saline. Protein was determined by the method of Lowry et al. [30], using BSA as standard.

**MTT reduction by isolated epithelial cells**

Cell viability and mitochondrial function were assessed by MTT reduction using a microplate reader as described with slight modification [31]. In a total volume of 150 µl in each well, cell suspension corresponding to 1 × 10⁶ cells/ml were taken, added 6 µl of 1.25 mM MTT and made up the volume with 25 mM phosphate buffered saline. MTT was dissolved in PBS and filtered thoroughly to dissolve the formazan. The plates were read on a multiwell scanning spectrophotometer (ELISA reader) using a test wavelength of 570 nm and reference wavelength of 630 nm. The amount of MTT formazan formed was calculated from the standard curve prepared using authentic MTT formazan (20–100 µM). Since succinate is a mitochondrial respiratory substrate, formation of MTT formazan in presence and absence of 1 mM succinate (final concentration) was tested.

**Conjugated diene formation**

Total lipids were extracted from various cell fractions as described [32] and evaporated using nitrogen. This was dissolved in 1 ml heptane and the absorbance was measured at 233 nm. The amount of conjugated diene formed in the sample was calculated using a molar absorption coefficient of 2.52 × 10⁴ [33].

**Polyunsaturated fatty acid analysis**

To an aliquot of the total lipid extract, 1 ml of methanol and 0.05 ml of concentrated HCl were added, a little nitrogen passed, and the tubes were kept at 70°C for 4 h. The tubes were cooled and extracted with 2 ml pentane, twice. Pooled pentane extract was dried using nitrogen. Fatty acid methyl esters were separated and quantitated
by gas chromatography using Pye Unicam 4550 gas chromatograph equipped with flame ionization detector and PU 4811 Spectraphysics integrator. The column used was glass column packed with 5% EGSS-X (Ethylene succinatemethyl siloxine polymer). Heptadecanoic acid was used as the internal standard.

**α-Tocopherol estimation**

α-tocopherol content of isolated epithelial cell fractions was measured using HPLC after extraction as described [34]. The tocopherol was detected using an UV detector at 294 nm. The concentration of α-tocopherol was determined using reference injections of standard α-tocopherol and by use of computing integrator. Tocopherol acetate was used as internal standard.

**SOD assay using MTT reduction method**

Microtiter plates (96 well) were used for assay of SOD activity [35]. Briefly, the assay mixture consist of 15 μl of 1 mM xanthine, 6 μl of 1.25 mM MTT, 30 μl of 15 mM/ml of XO, and 25 μl of cell homogenate (60–100 μg) and the volume was made up to 150 μl with 25 mM phosphate buffered saline pH 7.4, (Final concentrations of the reactants were 0.1 mM xanthine, 3 μU XO and 50 μM MTT and all the reagents were prepared in PBS). The mixture was incubated for 20 min at room temperature (30°C) and the reaction was terminated with the addition of 150 μl of DMSO, which helps to arrest the reaction and dissolve the MTT formazan crystals formed. Plates were shaken for a few minutes in an orbital shaker and read on a microtiter plate reader (Bio Rad Microtiter Reader model 450) using test wavelength of 570 nm and reference wavelength of 630 nm. One unit

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**Fig. 1.** MTT reduction by various isolated enterocyte fractions from monkey small intestinal epithelium in absence or in the presence of 1 mM succinate. Experimental details are described in the text. Data are expressed as mean ± SD of three independent experiments.

**Fig. 2.** Lipid peroxidation parameters in various enterocyte fractions from monkey small intestinal epithelium. A-conjugated diene; B-polyunsaturated fatty acids (linoleic acid-18:2 and arachidonic acid-20:4). Experimental details are described in the text. Data are expressed as mean ± SD of three independent experiments.
of SOD was defined as the amount of protein required to inhibit the MTT reduction by 50%.

Catalase assay

Various cell fraction homogenates containing catalase activity were estimated by measuring the change in optical density at 240 nm using \( \text{H}_2\text{O}_2 \) as substrate [36]. One unit is the activity that disproportionates \( \text{H}_2\text{O}_2 \) at the rate of \( 10^{-3} \) absorbance/second.

Glutathione S-transferase

The activity of GST in the cell homogenates was measured spectrophotometrically using the substrate CDNB as described [37]. One unit of enzyme is the amount required for conjugating 1 \( \mu \text{mole} \) of substrate with glutathione in 1 min.

Glutathione peroxidase

The activity of GPx was measured as described [38] and change in optical density at 340 nm was followed. One unit is the amount needed to oxidize 1 nmole of NADPH/minute.

Glutathione reductase

The activity of GSH reductase in the 10,000 \( \times \) g supernatant of cell homogenate was measured at 340 nm as described [39]. One unit is the amount of enzyme needed to convert 1 \( \mu \text{mole} \) of NADPH/minute.

Statistical analysis

Results are expressed as the mean \( \pm \) SD of three separate experiments.

RESULTS

Figure 1 shows the MTT reduction by cells from different fractions both in the presence and absence of the respiratory substrate, succinate. MTT reduction was considerably less in villus tip fractions (fractions 1 and 2) as compared to other cell fractions and this is also true in the presence of succinate. Oxidative stress was assessed in various cell fractions by quantitating the lipid peroxidation products, namely conjugated diene formation and polyunsaturated fatty acid depletion. As shown in Fig. 2, both villus tip cells (fractions 1 and 2) and crypt stem cells (fractions 8 and 9) showed an increased amount of conjugated diene and decreased level of polyunsaturated fatty acids as compared to middle cell fractions. This was further supported by the quantitation \( \alpha \)-tocopherol, which is a known antioxidant. As shown in Fig. 3, \( \alpha \)-tocopherol level was also low in both villus tip and crypt stem cells as compared to the middle fraction cells.
both villus and crypt cells showed significant decrease in this ratio [villus (fractions 1–3): 2.3; middle (fractions 4–6): 2.85; crypt (fractions 7–9): 2.55]. Superoxide dismutase and catalase activities were measured in various fractions and as shown in Fig. 4, activity of SOD was low and catalase was high in villus tip cells as compared to other cell fractions. There was a gradual increase in the activity of catalase from crypt to villus cells.

Certain glutathione utilizing enzymes offer protection from oxidative stress and these enzyme activities were measured in various cell fractions. As shown in Fig. 5, glutathione-S-transferase, glutathione peroxidase and glutathione reductase activities are high in villus tip cells as compared to other cell fractions and there is a gradual increase in the level of these enzymes from crypt to villus cells.

**DISCUSSION**

Apoptosis or programmed cell death is the process whereby cells are induced to activate their own death or cell suicide. Apoptosis occur in a wide variety of cell types and is required during the development of many tissues. Failure to negatively regulate apoptosis is associated with degenerative diseases, and failure to positively regulate apoptosis is associated with cancer [40]. In many models of apoptosis, cells are induced to die as a result of changes in environmental stimuli such as growth factors and hormones [41,42].

The process of apoptosis under in vitro conditions has been detected using various cell systems but it is often
unclear and difficult to detect apoptosis under in vivo conditions. The small intestinal epithelium offers a good system to study apoptosis under in vivo conditions due to differentiation, maturation and high turnover of epithelial cells. We have earlier shown using cells at different stages of differentiation from monkey small intestinal epithelium that the apoptotic cells are seen only in villus tip cells. This was established by DNA ladder pattern on agarose gel electrophoresis and Hoechst dye staining for chromatin condensation. The apoptotic processes in these cells were associated with decreased level of GSH due to increased efflux of this antioxidant from the cells. This, as well as the other published work on apoptosis prompted us to study the role of oxidative stress on the intestinal epithelial cell apoptosis.

Apoptosis appears to be the major mode of cell death when cells experience lethal oxidative insult from exposure to oxidants, including superoxide and hydrogen peroxide [43–45]. Interestingly, even cells that undergo apoptosis following nonoxidative insults, such as steroid treatment or viral infection, have been shown to accumulate lipid peroxides, which is an evidence of oxidative damage [46,47]. Decreased arachidonic acid (20:4) seen in villus cells could be due to formation of conjugated diene and this is supported by conjugated diene measurements on the villus cells as compared to crypt cells. Lipid peroxidation parameters clearly indicated that oxidative stress occurs in these cells but this was seen not only in villus tip cells but also in crypt stem cells. This was further supported by the low level of the lipid soluble antioxidant, α-tocopherol in both these types of cells as compared to middle cells, along with decreased ratio of α-tocopherol to polyunsaturated fatty acid. This suggests that there is increased oxidative stress both in villus tip and crypt stem cells.

In the mouse small intestine, apoptosis occurs rapidly after γ-irradiation and with the highest frequency in the stem cells (crypt cells) [48,25]. In contrast, Moss et al [49] showed that in the normal upper small intestine, apoptotic nuclei are seen only in the region of the villus tip by light microscopy whereas using the more sensitive TUNEL staining technique, apoptotic cells were also seen among crypt axis. Approximately two thirds of TUNEL stained apoptotic cells were present near the villus tip, about 20% near the crypt base and the remainder were seen between these regions. The data presented here also show that oxidative stress as shown by the lipid peroxidation is seen both in villus tip and crypt stem cells. This suggests that although apoptosis was seen only in villus tip cells, oxidative stress is observed in both villus tip and crypt stem cells. Indeed, epithelial cells near the lumen in normal small intestine are well known to show cell shrinkage and nuclear condensation when compared with mid-villus epithelium suggesting that they may be initiating the changes that lead to apoptosis.

Measurement of various antioxidant enzymes showed an increased level in villus tip cells as compared to other cells except SOD, which was lower in these cells. Increased activity of antioxidant enzymes in the villus tip cells may be an indication of the cell response to increased oxidative stress. It was found that glutathione-S-transferase gene expression was induced in steroid-mediated lymphocyte apoptosis [50] and in the present study increased activity of this enzymes was seen in apoptotic villus tip cells. Low activity of SOD in villus tip cells may be another contributing factor in apoptosis.

One interesting observation was the diminished mitochondrial function as assessed by MTT reduction in villus tip cells which was not regained even in the presence of succinate. This clearly shows that in apoptotic villus cells (fractions 1 and 2), mitochondrial function has been impaired. The accompanying paper presents data related to the functional and structural alteration to the villus cell mitochondria compared to crypt cell mitochondria. There are various reports on the role of mitochondria in apoptotic process. Increased mitochondrial permeability transition has been suggested to be an important factor in inducing apoptosis [51,52]. Thiol compounds, especially GSH protects the mitochondria from any damage, and depletion of mitochondrial GSH results in damage to mitochondria. Our earlier work has shown that villus tip cells show increased efflux of GSH and is associated with decreased level of mitochondrial GSH. This further supports defective mitochondria in apoptotic villus tip cells. In summary, this study has shown that mitochondrial function is impaired in the apoptotic villus tip cells and oxidative stress may play a role in this process.

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Oxidative stress and apoptosis


