APOPTOSIS IN THE MONKEY SMALL INTESTINAL EPITHELIUM: STRUCTURAL AND FUNCTIONAL ALTERATIONS IN THE MITOCHONDRIA

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Abstract—Our earlier studies have shown apoptosis in the villus tip cells of the monkey small intestinal epithelium. Because mitochondria have been implicated in the apoptotic process, this study looked at the function and lipid composition of mitochondria isolated from apoptotic villus tip cells and compared it with middle and crypt cells. Decreased MTT reduction and respiratory control ratio, increased swelling and altered mitochondrial enzyme activities were seen in the villus tip cell mitochondria when compared to other cells. The lipid composition of the villus tip mitochondria were different from the other mitochondria. A decrease in phosphatidylethanolamine and phosphatidylinositol and an increase in phosphatidic acid was seen in these mitochondria. Fatty acid composition analysis showed more unsaturated fatty acids in the free fatty acid and phospholipid fraction in villus tip cell mitochondria as compared to other cells. These studies suggest that in the monkey small intestinal epithelium, apoptotic process is associated with functional and structural alterations in the mitochondria. © 1999 Elsevier Science Inc.

Keywords—Monkey, Intestinal epithelium, Mitochondria, Apoptosis, Free radicals

INTRODUCTION

Apoptosis is a fundamental biological regulatory mechanism and is an essential process controlling tissue homeostasis in multicellular organisms by which damaged, infected, or neoplastic cells are continually eliminated [1]. It is an active and irreversible process in that cells activate their own death program. Apoptosis is characterized by cell shrinkage, nuclear condensation and fragmentation, plasma and nuclear membrane blebbing and appearance of apoptotic bodies [2,3]. Apoptosis can be induced by physiological processes and non-physiological stimuli including oxidative stress [4,5].

Structural and functional alterations in cells have been observed during apoptosis. The biochemical and molecular mechanisms involved in apoptosis have not yet been clearly elucidated. There are reports indicating a role for mitochondria in apoptosis [9–12]. Studies have also shown a partial depolarization of the mitochondrial membrane potential in apoptotic cells [6–8]. Oxidative stress induced apoptosis is prevented by Bcl-2 that is associated with the mitochondrial outer membrane [13]. It has also been shown that ATP generation by the mitochondria is necessary for a commitment to apoptosis [14].

Although there are a number of reports on apoptosis under in vitro conditions induced by various stimuli, it is difficult to study this process under in vivo conditions. Intestinal epithelial cells are ideal to study the apoptotic process under in vivo conditions because these cells originate in the crypt base and migrate towards the villus tip during their maturation and then undergo programmed death. These cells are either exfoliated into the lumen or engulfed by the surrounding macrophages. Our earlier studies using isolated cells of various stages of differentiation from the monkey small intestinal epithelium have shown that apoptotic cells are seen only in the villus tip cells [15]. It was also observed that these villus tip cells have a reduced level of the biologically important thiol, GSH, both in the cell and in the mitochondria as compared to the crypt and middle cells [15]. Our studies on the possible role of oxidative stress in these cells obtained from different stages of maturation, indicated that although oxidative stress is seen in both villus tip and crypt stem cells [16], mitochondrial function, as
assessed by MTT reduction, indicated a drastic reduction in the villus tip cells. These studies along with other published work with other cell systems have indicated a possible change in the mitochondrial structure and function during apoptosis. In the present study we have compared the structure and function of mitochondria obtained from enterocytes at different stages of maturation in order to understand the role of mitochondria in epithelial cell apoptosis.

**Materials and Methods**

Lipid standards, MTT (3-(4,5 dimethylthiazol-2-yl),2,5-diphenyl tetrazolium bromide), MTT formazan, superoxide dismutase, dimethyl sulfoxide, 2,6 dichlorophenol indophenol (DCIP), NADH, NAD, isocitric acid, ADP, NADP, succinate, p-iodonitrotetrazolium violet (INT), EGTA, dithiothreitol (DTT), HEPES and BSA, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents used were of analytical grade.

**Isolation of enterocytes**

Isolation of various epithelial cell fractions from the monkey small intestine and checking the purity of cell fractions are as described earlier [15].

**Isolation of mitochondria**

Because the yield of isolated cells in each fraction was not sufficient enough for mitochondrial preparation, Fractions 1+2, 3+4, 5+6 and 7+8+9 (indicated as I, II, III and IV respectively) were pooled and homogenized in ice cold medium containing 250 mM sucrose, 5 mM HEPES, 1 mM EDTA pH 7.4 with 0.01% BSA. Mitochondria were isolated by differential centrifugation as described [17]. Purity of the preparation was judged by the enrichment of the marker enzyme, succinate dehydrogenase [18].

**MTT Reduction by isolated epithelial cell mitochondria**

Mitochondrial function was assessed by MTT reduction using isolated mitochondria as described earlier [19].

**Mitochondrial enzyme assay**

Mitochondria isolated from various cell fractions of monkey small intestinal epithelium were used for the assay of the following enzymes. NADH dehydrogenase activity was measured spectrophotometrically by the rate of NADH-dependent 2,6-dichlorophenol indophenol (DCIP) reduction at 600 nm as described [20]. Activity of isocitrate dehydrogenase to both NAD [21] and NADP [22] was measured using threo-isocitrate as substrate by following the increase in absorbance at 340 nm. Succinate dehydrogenase activity was assayed using 2-(p-iodophenyl)-3-(p-nitropheno)-5-phenyl tetrazolium chloride (INT) as the electron acceptor [18]. Mitochondrial ATPase activity was also measured as described [23].

**Measurement of oxygen uptake**

Oxygen uptake was determined polarographically using a Clark type electrode in 2 ml of respiratory medium (225 mM Sucrose, 5 mM MgCl2, 10 mM KH2PO4, 20 mM KCl, 10 mM Tris, and 5 mM HEPES pH 7.4), containing 5 mM succinate. One mg/ml of mitochondrial protein was used. Oxygen uptake was stimulated with 0.3 mM ADP.

**SOD assay using MTT reduction method**

Microtiter plates (96 well) were used for assay of mitochondrial MnSOD activity [24]. Briefly, the mitochondrial suspension (60–100 μg) was preincubated with 5 mM sodium cyanide for 10 min and the assay mixture consisted of 15 μl of 1 mM xanthine, 6 μl of 1.25 mM MTT, 30 μl of 15 μU/ml of XO, 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 nU 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, that helps to arrest the reaction and dissolve the MTT formazan crystals formed. Plates were shaken for a few minutes 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 of SOD was defined as the amount of protein required to inhibit the MTT reduction by 50%.

**Mitochondrial swelling**

Mitochondrial swelling was determined by the decrease in absorbance at 540 nm as described [25].

**Lipid analysis**

Mitochondrial lipids were extracted as described [26]. The lower organic phase was evaporated to dryness,
resuspended in a small volume of chloroform:methanol (2:1 v/v), and used for lipid analysis. Neutral lipids were separated on silica gel G plates using the solvent system hexane-diethylether-acetic acid (80:20:1 v/v). Spots corresponding to standard were identified by iodine exposure and eluted. Cholesterol [27], diglycerides and triglycerides [28] were estimated as described. Free fatty acids were methylated and quantitated by gas chromatography after separation on a 5% EGSS-X column. Heptadecanoic acid was used as internal standard. Individual phospholipids were separated on silica gel H plates using the solvent system chloroform-methanol-acetic acid-water (25:14:4:2 v/v) [29] and quantitated by phosphate estimation after acid hydrolysis [30]. Individual amino phospholipids were also quantitated after derivatisation with fluorescamine and separation on silica gel H plates impregnated with 3% magnesium acetate using the solvent system chloroform-methanol-NH₄OH-water (60:40:5:2 v/v) [31]. Eluted individual spots were quantitated using Shimadzu SF 5000 spectrofluorometer with excitation at 395 nm and emission at 468 nm. Phosphatidic acid (PA) was quantitated after extracted lipids were spotted on silica gel G plates impregnated with 0.5 M oxalic acid and separated using the solvent system, chloroform-methanol-con.HCl (85:13:0.5 v/v) [32]. PA corresponding to standard was identified by iodine vapour exposure, eluted and quantitated by phos-

Fig. 1. MTT reduction by mitochondria obtained from various enterocyte fractions of monkey small intestinal epithelium in the absence or presence of 1 mM succinate. Experimental details are described in the text. I-represents villus fraction, II and III-represent middle cell fractions and IV-represents crypt stem cells. Each value represents mean ± SD of three independent experiments.

Fig. 2. Respiratory control ratio and swelling of mitochondria isolated from various enterocyte fractions from monkey small intestinal epithelium. (A) Respiratory control ratio. (B) Swelling was assessed by decrease in absorbance at 540 nm. Experimental details are described in the text. Each value represents mean ± SD of three independent experiments.
phate estimation after acid digestion [30]. Protein was determined by the method of Lowry et al. [33], using BSA as standard.

**Statistical analysis**

All data are presented as mean ± SD of three separate estimations.

**RESULTS**

The four fractions of cells used in this study comprise the villus tip cells (Fraction I), middle cells (Fraction II and III) and the crypt stem cells (Fraction IV). Mitochondria were isolated separately from these different fractions and their structural and functional aspects were compared. The functional aspects of mitochondria were tested by the reduction of the tetrazolium dye, MTT and measurement of the respiratory control ratio. As shown in Fig. 1A, MTT reduction was less in the mitochondria from Fraction I as compared to other fractions and this was also true even in presence of the respiratory substrate, succinate (Fig. 1B). Alteration in MTT reduction by the mitochondria indicates a disruption of respiration and this was checked by oxygen uptake measurements. As shown in Fig. 2A, respiratory control ratio was less than 1 for mitochondria from Fraction I indicating an uncoupling of respiration from oxidative phosphorylation, whereas the other fractions showed a ratio of more than 1. These results indicate that electron transport activity is reduced in villus cell mitochondria as compared to middle and crypt cells. This may be due to damage to mitochondria that was tested by the swelling of isolated mitochondria from various fractions. As shown in Fig. 2B, Fraction I mitochondria showed swelling as indicated by decreased absorbance at 540 nm whereas mitochondria from other fractions were normal. One of the possible factors responsible for mitochondrial damage is oxygen free radicals generated by the mitochondria as well as other cellular components.

Mitochondria are normally protected from oxidative stress by the presence of MnSOD in this organelle. The activity of this enzyme was measured in isolated mitochondria from all fractions and as shown in Fig. 3, MnSOD activity was considerably less in Fraction I mitochondria as compared to other fractions. Some of the mitochondrial enzymes were measured in mitochondria obtained from various fractions and as shown in Fig. 4, except isocitrate dehydrogenase (NADP specific), all other enzymes studied showed a significant decrease in activity in Fraction I mitochondria as compared to other fractions.

Lipids are important constituents of mitochondrial membranes and alterations in their composition may be responsible for altered mitochondrial function. Phospholipids were analysed in mitochondria obtained from var-
ious cell fractions. As shown in Table 1, among the phospholipids, phosphatidylethanolamine and phosphatidylinositol were decreased in Fraction I as compared to other fractions. The level of phosphatidylcholine, phosphatidylserine, sphingomyelin and cardiolipin were not altered significantly among various fractions. There is a gradual small increase in the content of lysoPC and lysoPE from Fraction I to Fraction IV. Our earlier work has indicated an active conversion of enterocyte mitochondrial PE to phosphatidic acid in response to various stimuli, including oxygen free radicals and in this study the level of PA was measured in mitochondria obtained from various fractions. It was seen that Fraction I had the highest level of this bioactive lipid (29.85 ± 1.20 nmol/mg protein) and the level was lower but more or less same in the other fractions (II: 16.3 ± 0.8, III: 14.25 ± 1.0, and IV: 12.8 ± 1.0). Neutral lipids were high in mitochondria from Fraction IV that comprises mainly crypt stem cells as compared to other fractions (Table 2). Free fatty acid analysis indicated a higher content in Fraction IV as compared to other fractions (Table 3). Interestingly in villus fraction mitochondria, unsaturated fatty acids linoleic (18:2) and arachidonic acid (20:4) were high whereas in the crypt cell mitochondria, saturated fatty acids were high. Analysis of the fatty acid composition of the phospholipids indicated a higher arachidonic acid (20:4) level in villus cell mitochondrial phospholipids as compared to other fractions (Table 4). Other fatty acids showed a slight increase in crypt cell mitochondrial phospholipids.

**DISCUSSION**

An increasing number of molecular events have been shown to regulate cell death by apoptosis. Most of these studies have been carried out using in vitro systems and very little is known on the process of apoptosis under in vivo conditions. Intestinal mucosal epithelial cells offer an ideal system to study the apoptotic process in vivo. Our earlier studies using isolated monkey small intestinal villus, middle and crypt cells have shown that apoptotic process is seen only in villus tip cells [15,16]. This was associated with GSH efflux and decreased GSH content in the villus apoptotic cells as well as in mitochondria, and mitochondrial function when assessed by MTT reduction was considerably decreased in villus tip cells as compared to crypt stem cells. Because these studies indicated a possible derangement of mitochondrial function in apoptotic villus tip cells, the present study has looked at the structural and functional aspects of mitochondria obtained from apoptotic villus cells (Fraction I) in comparison with middle and crypt cell mitochondria (Fractions II, III, and IV).

This study indicated that apoptosis in the villus tip cells is associated with 1) mitochondrial swelling and an altered respiratory control ratio, 2) decrease in the level of MnSOD, 3) decrease in the capacity to reduce the tetrazolium dye, MTT, even in the presence of mitochondrial respiratory substrate, succinate, and 4) low activities of mitochondrial enzymes. In addition to this, alteration in lipid composition was also seen. Recent studies

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**Table 1. Phospholipid Composition of Monkey Small Intestinal Epithelial Cell Mitochondria**

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>Fractions</th>
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<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Phosphatidylcholine (PC)</td>
<td>74.80 ± 3.00</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (PE)</td>
<td>58.40 ± 2.90</td>
</tr>
<tr>
<td>Lyso PC</td>
<td>6.50 ± 1.00</td>
</tr>
<tr>
<td>Lyso PE</td>
<td>9.30 ± 0.75</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>6.81 ± 0.50</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>4.17 ± 0.40</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>12.30 ± 0.80</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>24.87 ± 1.40</td>
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</tbody>
</table>

Each value represents mean ± SD of three separate estimations.

**Table 2. Neutral Lipid Composition of Monkey Small Intestinal Epithelial Cell Mitochondria**

<table>
<thead>
<tr>
<th>Neutral lipids</th>
<th>Fractions</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>39.35 ± 2.30</td>
</tr>
<tr>
<td>Cholesterol ester</td>
<td>6.50 ± 1.00</td>
</tr>
<tr>
<td>Diglyceride</td>
<td>10.70 ± 1.00</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>22.70 ± 2.60</td>
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</tbody>
</table>

Each value represents mean ± SD of three separate estimations.
have suggested an important role for mitochondria in the apoptotic process. Members of the Bcl-2 family of proteins are located preferentially in the outer mitochondrial membrane [34], and it has been shown that the mitochondria-enriched cytoplasmic fraction by itself is capable of inducing nuclear apoptosis [35]. Reactive Oxygen Species (ROS) such as superoxide anion are normally generated as byproducts of mitochondrial respiration [36], and evidences suggest that reactive oxygen species (ROS) may participate as effector molecules in programmed cell death [37,38]. Zamzami et al. have shown that assessment of mitochondrial ROS generation provides an accurate picture of programmed cell death-mediated lymphocyte depletion [7]. It has been shown that mitochondrial permeability transition plays a role in apoptosis [10,39–42], and it can be initiated by oxidative stress [41,43].

In this study we have seen increased swelling of mitochondria in villus tip apoptotic cells accompanied by a decreased level of MnSOD and the previous study [15] showed a decreased GSH level in the mitochondria. Membrane permeability transition induced by oxidative stress can be aggravated if the level of antioxidants are low. Thus the villus tip apoptotic cell mitochondria may have compromised mitochondrial defenses against oxidative stress and this may be responsible for the decrease in RCR and mitochondrial enzyme activities seen in these cells. We also observed that mitochondrial ATPase activity was drastically decreased in Fraction I. This correlates well with the finding that mitochondria in apoptotic cells retain a portion of their transmembrane potential, raising the possibility that like cytochrome c, the mitochondrial ATPase is also defunctionalized in some way as cells undergo apoptosis [44].

Structural alterations in the mitochondria may lead to a functional impairment in this organelle. Lipids being important constituents of the mitochondrial membrane, an alteration in their composition may have a great effect on mitochondrial function. Fraction I (villus tip cell) mitochondria showed a decreased content of PE and an increased level of PA as compared to other fractions. Our earlier work has shown that enterocyte mitochondria contain a phospholipase D that preferentially hydrolyzes PE to PA when stimulated by oxygen free radicals, calcium or polyamines [45–47]. Decreased level of MnSOD and increased degradation of PE to PA by PLD in Fraction I mitochondria suggest that these mitochondria undergo lipid alterations possibly due to oxidative stress. These lipid alterations in turn may lead to functional alterations seen in these mitochondria. PA is a bioactive lipid and is involved in many signaling systems in the cell [48]. Recently it has been demonstrated that PA inhibits sarcoplasmic reticulum ATPase activity [49]. In our study too, we find increased PA formation accom-

### Table 3. Total and Individual Free Fatty Acid Content of the Monkey Small Intestinal Epithelial Cell Mitochondria

<table>
<thead>
<tr>
<th>Fatty acids (nmol/mg protein)</th>
<th>Fractions</th>
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<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>12:0</td>
<td>2.88 ± 0.10</td>
</tr>
<tr>
<td>14:0</td>
<td>1.64 ± 0.07</td>
</tr>
<tr>
<td>16:0</td>
<td>15.35 ± 1.00</td>
</tr>
<tr>
<td>18 and 18:1</td>
<td>16.44 ± 1.30</td>
</tr>
<tr>
<td>18:2</td>
<td>15.07 ± 0.80</td>
</tr>
<tr>
<td>20:4</td>
<td>21.01 ± 1.20</td>
</tr>
<tr>
<td>Total</td>
<td>72.38 ± 4.50</td>
</tr>
</tbody>
</table>

Each value represents mean ± SD of three separate estimations.

### Table 4. Fatty Acid Composition of Phospholipids in the Monkey Small Intestinal Epithelial Cell Mitochondria

<table>
<thead>
<tr>
<th>Fatty acids (nmol/mg protein)</th>
<th>Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>14:0</td>
<td>7.25 ± 0.60</td>
</tr>
<tr>
<td>16:0</td>
<td>129.50 ± 3.70</td>
</tr>
<tr>
<td>18 and 18:1</td>
<td>75.70 ± 4.30</td>
</tr>
<tr>
<td>18:2</td>
<td>190.31 ± 6.80</td>
</tr>
<tr>
<td>20:4</td>
<td>48.50 ± 2.90</td>
</tr>
</tbody>
</table>

Each value represents mean ± SD of three separate estimations.
panied by decreased ATPase activity in the villus cell mitochondria.

Thus the study suggests that a change in the mitochondrial membrane phospholipids may have a role in early cell death. Because phospholipids are the major structural components of membranes surrounding most intracellular organelles, such as lysosomes, endoplasmic reticulum, mitochondria and nuclei, a major change in phospholipid composition could be expected to cause deformation and porosity of such membranes, thus resulting in an inflow of deoxyribonucleases into the nucleus. This could cause the characteristic cleavage and laddering of chromosomal DNA as observed during apoptosis.

In conclusion, this study has demonstrated an altered lipid composition in mitochondrial membrane and functional alterations in villus apoptotic cell mitochondria of the monkey small intestinal epithelium. These structural and functional alteration to mitochondria in villus tip cells may be responsible for the apoptosis seen in these cells.

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Mitochondrial changes and apoptosis


