Surgical stress-induced alterations in retinoid metabolism in the small intestine: role of oxygen free radicals

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Abstract

Oxidative stress in the small intestine can result in altered cell proliferation, migration, and differentiation of villus-crypt cells. Retinoid metabolism is recognized as an important mediator of cellular differentiation in the intestine. This study examined the effect of oxidative stress in retinoid metabolism in a surgical stress model. Surgical stress was performed by handling the intestine as done during laparotomy. Villus-crypt cells were isolated at different time periods and various retinoid concentrations in the cell homogenate and the retinoic acid forming enzymes were quantitated using HPLC. Surgical stress resulted in altered retinoid levels in various cell populations in the small intestine at 1 and 12 h. The activity of alkaline phosphatase and retinal oxidase was also altered at these time points and all these changes were prevented by inhibiting superoxide generation using xanthine oxidase inhibitor, allopurinol. These studies suggest that alterations seen in enterocytes during surgical stress may be mediated by changes in retinoid metabolism.

Keywords: Retinoids; Enterocytes; Surgical stress; Oxidative stress; Alkaline phosphatase; Retinal oxidase; Proliferation; Differentiation

The intestinal epithelium undergoes a continuous renewal process and has cells at various stages of differentiation. The integrity of the intestinal epithelium is critical to health [1] and any damage to the cells can affect proliferation as well as differentiation, leading to altered cell population and functional changes in the intestine. The intestine acts as a barrier to the luminal contents which include bacteria and endotoxins. The gut barrier is altered in certain pathological conditions such as shock, trauma or surgical stress, leading to bacterial or endotoxin translocation from the gut lumen into the systemic circulation [2]. This has been implicated in post-operative complications such as systemic inflammatory response syndrome (SIRS)\(^1\) and multiorgan failure (MOF) [3]. Oxygen free radicals are known to play an important role in gut epithelial damage, which may alter gut barrier function and facilitate bacterial translocation and release of endotoxin. Our earlier work using a rat model has shown that laparotomy and intestinal handling, which could occur during any abdominal surgery, can result in increased intestinal permeability and oxidative stress in the enterocytes [4]. Oxidative stress in this system is mainly due to the generation of superoxide anion by xanthine oxidase and the damage is reversible with time [5]. The integrity and homeostasis of the intestinal mucosa are largely dependent on the continued

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\(^1\) Abbreviations used: SIRS, systemic inflammatory response syndrome; MOF, multiorgan failure; BSA, bovine serum albumin; EGTA, ethylene glycol-bis-(\(\beta\)-aminoethyl ether) \(\text{N,N',N''}\text{-}\text{tetraacetic acid}; \text{DTT}, \text{dithiothreitol}; \text{NAD}, \text{nicotinamide adenine dinucleotide; ALP, alkaline phosphatase; RA, retinoic acid; RAR, retinoic acid receptor; GI, gastrointestinal tract.}
proliferation, migration, and differentiation of the crypt cells [6]. These multipotent stem cells located near the base of each intestinal crypt divide to produce daughter stem cells and more rapidly proliferating transit cell. Transit cells in turn undergo a number of rapid cell divisions and differentiate into mature epithelial cells, migrate to the villus, and either die or are extruded into the lumen [7].

Vitamin A (retinol) plays an important role in the control of differentiation and proliferation of various epithelia of the body [8]. Absence of vitamin A can lead to uncontrolled proliferation of epithelial stem cells that fail to differentiate to the normal phenotype in many lining epithelia. Retinol and its metabolites are essential for growth and cell differentiation, particularly of epithelial tissue [9]. Restricted availability of retinol to the intestinal epithelial cells has been shown to result in impaired proliferation control of these cells [10,11]. To develop its biological function in these cells, retinol has to undergo cytosolic oxidation via retinal to (all-trans) retinoic acid [12,13], which is an active ligand for retinoic acid receptors [14,15]. Retinoic acid is the most physiologically active metabolite of vitamin A that modulates biological processes involved in embryogenesis, skeletal development, cellular differentiation, and growth, also it can modulate programmed cell death, may play an important role in the adapting intestine, and it alters intestinal adaptation after partial small bowel resection [16]. Since our earlier study had suggested that surgical stress leads to altered cell proliferation, migration, and differentiation of enterocytes [17] and retinoids play an important role in cell proliferation and differentiation, we hypothesize that changes in retinoid metabolism may play a role in mediating alterations seen in the villus-crypt cells after surgical stress. This was investigated in an animal model, using laparotomy and intestinal handling to induce surgical stress.

Materials and methods

Bovine serum albumin (BSA), ethylene glycol-bis-(B-aminoethyl ether) N,N,N’N’-tetaacetic acid (EGTA), dithiothreitol (DTT), nicotinamide adenine dinucleotide (NAD), allopurinol, p-nitrophenyl phosphate, trans-retinol, all-trans-retinal, and all-trans-retinoic acid were all obtained from Sigma Chemical (St. Louis, USA). All other chemicals used were of analytical grade.

Animals

Adult Wistar rats of both sexes (200–250 g) exposed to a daily 12 h light and dark cycle and fed water and rat chow ad libitum were used for the study. The rats were randomly divided into seven groups, group I (n = 4), control (laparotomy alone without intestinal handling), group III (n = 4), V (n = 4), and VII (n = 4) different time periods such as 1, 12, and 24 h after surgical stress (laparotomy with intestinal handling), group II, allopurinol control (laparotomy alone without intestinal handling after allopurinol treatment), group IV (n = 4) and VI (n = 4), 1 and 12 h after surgical stress (laparotomy with intestinal handling after allopurinol treatment). This study was approved by the Animal Experimentation Ethics Committee of the Institution.

Induction of surgical stress in rats

Surgical stress was carried out as described [18]. Briefly, overnight fasted rats were anaesthetized and the abdominal wall was opened by a vertical incision of approximately 4 cm. The intestine was gently moved and the ileocaecal junction was identified. The intestine was handled along its entire length from the ileocaecal junction proximally, simulating the “inspection” that occurs in a clinical setting. The intestine was then replaced back in the abdominal cavity and the whole process was completed within 1–2 min. Following this, the abdominal wall was sutured. The animals were killed by decapitation at 1, 12, and 24 h after the surgical procedure. This experimental model has been well established in our laboratory and many studies have been carried out using this model. For inhibition of XO activity, rats were given intraperitoneal injection of allopurinol (100 mg/kg body weight), 1 h before induction of surgical stress. Following this, the animals were sacrificed and various enterocyte populations were isolated from the intestine.

Isolation of villus and crypt cells from the small intestine

Care was taken both during cell isolation and retinoid extraction to avoid any light and all procedures were carried out under dim light. Following surgical stress, abdomen was opened, entire length of the small intestine was removed and washed gently with ice-cold physiological saline, pH 7.4, containing 1 mM DTT. Enterocytes of various stages of maturation (villus to crypt) were isolated by the metal chelation method as described [19]. Briefly, the intestine was filled with solution A (1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH2PO4, and 5.6 mM Na2HPO4, pH 7.3), clamped at both ends and incubated at 37°C for 15 min in the beaker containing solution A. Following incubation, the luminal contents of the intestine were discarded and the intestine was filled with solution B (phosphate-buffered saline, pH 7.3, containing 1.5 mM EDTA and 0.5 mM DTT), and incubated at 37°C for different time intervals of 4, 2, 2, 3, 4, 6, 7, 10, and 15 min in the beaker containing solution B. At the end of each time period, the incubated solution containing cells was collected in separate tubes. All these nine fractions were pooled into three. The first three fractions being the villus, the next three
fractions, the middle cells, and the last three fractions being the crypt cells. These cell fractions were centrifuged at 900 g for 5 min, washed with Krebs–Hensleit buffer, pH 7.4, containing 5 mM glucose and 2.5 mM calcium. Separated villus, middle, and crypt cells were identified by assaying the marker enzyme for differentiation, alkaline phosphatase (ALP) [20].

Retinoid extraction and quantitation by HPLC

The suspended cells were homogenized and the homogenate corresponding to approximately 2 mg protein was mixed with an equal volume of 100% ethanol and 0.025 × volume of 0.1 N HCl. Neutral and acidic retinoids were extracted twice with 3 × volume of hexane. Extracted fractions were dried under nitrogen and reconstituted in 100% ethanol for HPLC separation. Retinoids were separated on a Shim-pack CLC-SIL silica column running at 1 ml/min using the mobile phase (hexane:dioxane:acetic acid, 92:8:0.1). Retinol, retinal, and retinoic acid were monitored at 350 nm [8]. Quantitation was performed by relating the area of the peak to areas obtained by the analysis of known quantities of retinoid standards and expressed as pmol/mg protein. The recovery of these retinoids by this method was around 85%.

Measurement of enzyme involved in retinoic acid formation

Subcellular fractionation of various cell populations of enterocytes was carried out by differential centrifugation and cytosol was prepared. This was used for assay of enzymes involved in retinoic acid formation. Retinal dehydrogenase activity was estimated by measuring the retinoic acid formed from retinal. The reaction mixture consisted of cytosolic protein corresponding to approximately 1 mg, 200 μM nicotinamide adenine dinucleotide (NAD) and 0.5 μM retinal (in ethanol) (all final concentration) in a total volume of 0.2 ml. This was incubated at 37 °C for 20 min and the reaction was stopped by the addition of ethanol, followed by extraction and quantitation by HPLC as described above. Cytosolic incubations were also carried out in the absence of NAD to look for retinal oxidase activity. Specific activity was expressed as picomoles of all-trans-retinoic acid formed per minute per milligram of protein.

Protein estimation

Protein was estimated by Lowry’s method using bovine serum albumin as standard [21].

Statistical analysis

Data are expressed as means ± SD from a minimum of four animals with duplicate estimations. The Mann–Whitney non-parametric test was used for tests of significance of differences between groups. A probability of less than 0.05 was accepted as significant. Statistical calculations were done using SPSS (version 9) software.

Results and discussion

The turnover of intestinal epithelial cells is a finely regulated process extending from undifferentiated crypt stem cells to terminally differentiated villus cells. It has been shown that retinoid metabolites are essential for growth and cell differentiation, particularly of intestinal epithelium [9]. The conversion of β-carotene to vitamin A in the small intestine provides the first evidence that these carotenoids are the direct precursors of vitamin A in animals [22]. Vitamin A is stored in the liver as retinyl esters and when needed, is exported into the blood, where it is carried by retinol binding protein for delivery to other tissues. β-Carotene in the diet can be converted to two molecules of retinal in the small intestine by cleavage, specifically at its central double bond, catalysed by β-carotene, 15,15'-monooxygenase, and the retinal formed can be further metabolized either by reduction to retinol or retinoic acid by retinal reductase and retinal dehydrogenase, respectively [23]. The formation of retinoic acid, the active metabolite of retinol, is catalysed by two enzymes, retinal dehydrogenase (NAD dependent) and retinal oxidase (NAD independent) (Fig. 1). Retinoid levels in various populations of enterocytes lining the small intestine of normal rats and rats that underwent surgical stress were studied and compared. In the normal enterocytes, the retinol level was nearly 10-fold as compared to retinal and retinoic acid. It was found that the normal proliferating crypt cells have increased level of retinol, retinoic acid, and retinal as
compared to villus and middle cells. Surgical stress resulted in dynamic alterations in the level of retinoids in all the three cell populations of enterocytes (Figs. 2–4).

Our earlier study has shown that surgical stress increases the activity of xanthine oxidase enzyme, which is responsible for superoxide anion production [5] and also it can result in alterations in the intestinal epithelium as shown by decreased cell viability and yield in the crypt cells as compared to villus and middle cells. This was associated with cytoskeletal changes as well as altered enterocyte proliferation resulting in differential distribution [16]. At the mitochondrial level, altered mitochondrial functions such as decreased respiratory control ratio, increased superoxide generation, and induction of permeability transition in the crypt cells were observed as compared to villus and middle cells [24]. These changes were time dependent and reversed to normal pattern by 24 h [5]. Since retinoids, in particular retinoic acid, play an important role in cell proliferation, this study looked at changes in retinoid levels in various cell populations of the intestinal epithelium following surgical stress and also the enzyme involved in the formation of retinoic acid.

The villus cells of the intestinal epithelium showed increased level of retinol and decreased level of retinal and retinoic acid at 1 h following surgical stress and with time (24 h) the retinol and retinal levels came back to control, whereas the level of retinoic acid remained elevated (Figs. 2A–C). There was an increase in retinol and retinal, and decreased level of retinoic acid in the middle cells after surgical stress and by 24 h, the pattern was similar to control (Figs. 3A–C). Crypt cells showed decrease in retinoic acid at 1 h without any alterations in retinol and retinal levels following surgical stress (Figs. 4A–C). The maximum increase in retinoic acid was seen at 12 h following surgical stress as compared to other time periods and this was true with all the cell populations. All these alterations in retinoid levels were prevented by inhibiting XO activity by allopurinol treatment prior to surgical stress (Figs. 2–4), indicating that superoxide generation from XO is probably the initiating signal for these changes in retinoid levels. The movement of cells from the crypt to the villus tip in the rat intestine is completed in 2–5 days and terminates with cells being removed by apoptosis and/or by exfoliation [25]. Our earlier study has shown that surgical stress in the intestine induced alterations in the normal physiology. The crypt cells bear the brunt of the damage and dynamic changes in cell migration patterns also occur. However, the presence of stem cells preserves the ability

Fig. 2. Retinoid level in villus cells isolated from control and at various time periods after surgical stress. Each value represents mean ± SD of four separate experiments with duplicate estimation. *P < 0.05 as compared to control and #P < 0.05 as compared to 1 and 12 h without allopurinol treatment.

Fig. 3. Retinoid level in middle cells isolated from control and at various time periods after surgical stress. Each value represents mean ± SD of four separate experiments with duplicate estimation. *P < 0.05 as compared to control and #P < 0.05 as compared to 1 and 12 h without allopurinol treatment.

Fig. 4. Retinoid level in crypt cells isolated from control and at various time periods after surgical stress. Each value represents mean ± SD of four separate experiments with duplicate estimation. *P < 0.05 as compared to control and #P < 0.05 as compared to 1 and 12 h without allopurinol treatment.
of regeneration and healing, and the reversibility seen after damage is probably facilitated by the rapid movement of new cells to replace the damaged cells. All these dynamic changes in the retinoids seen following surgical stress suggest that retinoids might play an important role in cell proliferation and differentiation.

Alterations in the retinoid levels in villus-crypt axis may lead to changes in differentiation patterns of these cells in the small intestine. Alkaline phosphatase (ALP) is the differentiation marker enzyme used to identify cells along the villus-crypt axis and this enzyme has been shown to be highly susceptible to free radicals [26,27]. ALP activity was significantly decreased in villus, middle, and crypt cells at 1 h after surgical stress compared to control and this was prevented by allopurinol treatment. However, at the 12 h time point, ALP activity increased in the villus and middle fractions and this was brought to normal pattern by allopurinol treatment, by 24 h the activity was reversed to control following surgical stress (Figs. 5A–C).

To understand the physiological role of retinoids in the enterocyte proliferation and differentiation, it is crucial to know the formation of retinoic acid (RA) in the small intestinal enterocytes. The biological function of retinoids in these cells is mediated by their cytosolic oxidation to all-trans-retinoic acid [12,13], which is an active ligand for retinoic acid receptors (RARs) [14,15]. Increased formation of retinoic acid was seen in the normal crypt cells due to higher activity of retinal oxidase or retinal dehydrogenase, the enzymes responsible for the conversion of retinal to retinoic acid. Enzyme activity in presence and absence of NAD was studied and it was found that the enzyme responsible for this conversion in the rat intestinal epithelium was not dependent on NAD, suggesting that this enzyme is an aldehyde oxidase. Hence, it appears that rat enterocyte cytosol contains retinal oxidase rather than retinal dehydrogenase. In the normal intestine, this enzyme was more active in crypt cells as compared to villus and middle cells. Following surgical stress, the enzyme activity is significantly decreased in all the three populations of enterocytes at 1 h. However, the activity increased nearly 4-fold in villus and middle cells, and 2-fold in crypt cells by 12 h and these changes were prevented by inhibiting the enzyme XO by allopurinol treatment before surgical stress. By 24 h, the activity partially returned to control in villus and middle cells, but in crypt cells it remained high as 12 h (Figs. 6A–C). Formation of retinoic acid has been shown earlier using total enterocytes [28]. Studies have also shown a role for retinoic acid in stimulating early
cellular proliferation in the adaptation of the small intestine after partial resection [16]. Retinoic acid has dual activities, both inhibiting proliferation and increasing proliferation [10]. RA brings about cell proliferation by binding to specific RA receptors which increases the transcription. There are different isomers of retinoic acid and these isomers can exert their effect only if the corresponding nuclear receptors are present [29]. The involvement of receptors for retinoic acid isomers in signal transduction pathways emphasizes the importance of sufficient retinoic acid production for controlled cell growth. Retinoic acid, the physiologically active metabolite of vitamin A, also plays an important role in controlling progression to carcinogenesis in a variety of cancers including squamous cell carcinoma of the neck, skin cancer, and acute promyelocytic leukemia and has been used in the treatment of these malignant conditions [30].

In summary, this study has demonstrated alterations in the retinoid levels in different cell populations of the rat small intestinal epithelium following surgical stress. The reversal of these changes by inhibition of XO suggests that superoxide generation from this enzyme probably acts as an upstream signalling mediator in this process. The present study provides new insights into the role of retinoids in cell proliferation and differentiation during surgical stress and also the susceptibility of retinoids to superoxides generated by the xanthine oxidase activity during the process. These changes in retinoid metabolism probably supports a role for gastrointestinal tract (GI) in post-surgical complications such as systemic inflammatory response syndrome and multiorgan failure where oxygen free radicals also play an important role.

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