Effect of surgical manipulation of the rat intestine on enterocyte populations

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Background. The intestine is susceptible to operations at remote locations, and the barrier function is altered during intestinal manipulation, leading to bacterial or endotoxin translocation into the systemic circulation. One of the mainstays for the maintenance of the integrity of the barrier function is epithelial cell proliferation and migration. The present study looked at the effect of gut manipulation after laparotomy on different cell populations of the intestinal epithelium.

Methods. Surgical manipulation of the gut was performed by opening the abdominal wall and handling the intestine, as is done during laparotomy. Villus and crypt cells were isolated at different time periods after gut manipulation, and mitochondria were prepared from isolated enterocytes. The effects of surgical manipulation on enterocytes and isolated mitochondria were studied.

Results. Mechanical manipulation of the gut resulted in alterations in the intestinal epithelium, as shown by decreased cell viability and yield in the crypt cells. The alterations were associated with actin reorganization, as well as with altered cell proliferation and adenosine deaminase activity. At the mitochondrial level, altered mitochondrial function, such as decreased respiratory control ratio, increased 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide reduction, and induction of permeability transition in the crypt cells, was observed. These alterations were maximal 1 hour after surgical manipulation and partially recovered to normal by 24 hours.

Conclusions. Mechanical manipulation of the gut that occurs during any abdominal operation induces alterations in the intestine, both at the cellular and the subcellular levels. The crypt cells bear the brunt of the damage, and the reversibility of the damage is possibly brought about by increased proliferation and movement of the cells. (Surgery 2001;130:479-88.)

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base of each intestinal crypt divide to produce a daughter stem cell and a 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. Since the intestinal epithelium has high turnover of cells and consists of enterocytes at various stages of differentiation, the present study looked at the effect of surgical manipulation of the gut on different cell populations of the intestinal epithelium.

**MATERIAL AND METHODS**

Adenosine diphosphate (ADP), bovine serum albumin (BSA), ethylene glycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), dithiothreitol (DTT), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were all obtained from Sigma Chemical Co (St Louis, Mo). Tritiated thymidine (740 G Bqm/mol) was obtained from Bhaba Atomic Research Centre (Bombay, India). All other chemicals used were of analytic grade.

**Animals.** Adult Wistar rats of both sexes (200-250 g) exposed to a daily 12-hour light-dark cycle and given free access to water and rat chow were used for the study. This study was approved by the Animal Experimentation Ethics Committee of the institution.

**Induction of surgical manipulation.** The surgical manipulation of the gut was carried out as previously described. Briefly, overnight-fasted rats were anesthetized by intraperitoneal injection of ketamine (50 mg/kg body weight). The abdominal wall was opened by a vertical incision of approximately 4 cm and the intestine handled along its entire length from the ileocecal junction proximally, simulating the “inspection” that occurs in a clinical setting. The intestine was then replaced in the abdominal cavity. The whole process was completed within 1 to 2 minutes. The abdominal wall was then sutured, and the animals were killed by decapitation 1, 2, 4, 12, and 24 hours after the surgical procedure. For sham control, the same procedures previously described were followed, except that the intestine was not handled.

**Isolation of villus and crypt cells from the small intestine.** The whole length of the small intestine was washed gently with cold physiologic saline solution containing 1 mmol/L DTT. Enterocytes of various stages of maturation (villus to crypt) were isolated by the metal chelation method as previously described. Briefly, the intestine was filled with solution A (1.5 mmol/L KCl, 96 mmol/L NaCl, 27 mmol/L sodium citrate, 8 mmol/L KH₂PO₄, and 5.6 mmol/L Na₂HPO₄, pH 7.3), clamped at both ends, and incubated at 37°C for 15 minutes. After incubation, the luminal contents of the intestine were discarded and the intestine was filled with solution B (phosphate-buffered saline solution, pH 7.3, containing 1.5 mmol/L EDTA and 0.5 mmol/L DTT) and incubated at 37°C for different time intervals of 4, 2, 2, 3, 4, 6, 7, 10, and 15 minutes. At the end of each period, the incubated solution containing cells was collected in separate tubes. All 9 of these fractions were pooled into 3 fractions. The first 3 fractions were the villus cells, the next 3 were the mixed cells, and the last 3 were the crypt cells. These cell fractions were centrifuged at 900g for 5 minutes and washed with Krebs-Hensleit buffer, pH 7.4, containing 5 mmol/L of glucose and 2.5 mmol/L of calcium. Separated villus, mixed, and crypt cells were identified by assaying the marker enzyme for differentiation, alkaline phosphatase. Mitochondria were prepared from separated cells as previously described.

**Cell viability and yield.** Cell viability was studied by the dye-exclusion method, using trypan blue. Cell counts of various fractions were made after isolation. The proportion of the individual cell population was expressed as percentage of total isolated cells.

**Cellular uptake of tritiated thymidine.** One milliliter of cell suspension corresponding to 1 to 2 x 10⁶ cells was incubated with 0.5 μCi ³H thymidine at 25°C for 20 minutes. At the end of incubation, 100% trichloroacetic acid was added to make a final concentration of 20%, kept at 4°C for 10 minutes, and centrifuged. To the precipitate, 1 mL of 5% TCA was added, kept in the boiling water bath for 30 minutes, and centrifuged. The supernatant was transferred to scintillation vials for counting. Thymidine incorporation was expressed as disintegrations per minute (dpm)/10⁷ cells.

**Actin polymerization.** Filamentous and unpolymerized actin were quantified by DNAase inhibition assay as previously described.

**Biochemical estimations.** The activity of the alkaline phosphatase was assayed as previously described. The assay mixture contained 0.5 μmol/L p-nitrophenol phosphate, 50 μmol/L Tris/HCl buffer, pH 9, 0.5 μmol/L MgCl₂, and enzyme in a total volume of 0.5 mL. It was incubated at 37°C for 15 minutes, and the reaction was stopped by the addition of 2.5 mL of 1N NaOH. The yellow color developed as a result of the release of p-nitrophenol was measured at 405 nm. Adenosine deaminase activity was measured as described. The assay mixture contained 0.1 μmole of adenosine, 0.3 mmol/L of potassium phosphate.
buffer, pH 6.5, and enzyme in a total volume of 3 mL. This gives an initial rate indicated by 0.010 per minute decrease in optical density (260 nm, 1-cm light path) at 25°C. Protein estimation was done by using bovine serum albumin as the standard.\textsuperscript{15}

**Mitochondrial function.** Polarographic determination of oxygen uptake by isolated mitochondria was performed using a Clark-type electrode in 2 mL of respiration medium (225 mmol/L sucrose, 5 mmol/L KH$_2$PO$_4$, 20 mmol/L KCl, 10 mmol/L Tris, and 5 mmol/L Hepes pH 7.4) containing 5 mmol/L succinate as respiratory substrate. Oxygen uptake was stimulated with 0.3 mmol/L ADP. Mitochondrial swelling was determined by the decrease in absorbance at 540 nm.\textsuperscript{16} The MTT reduction by mitochondria was done using a microplate reader as described.\textsuperscript{17} Briefly, mitochondrial suspension (150 to 200 µg protein) was added to wells containing 6 µL of 1.25 mmol/L MTT, and the volume was made up to 150 µL with 25 mmol/L phosphate-buffered saline. Plates were incubated at 37°C for 20 minutes, followed by the addition of 150 µL of DMSO, and

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**Fig 1.** Cell viability (A) and yield (B) of different enterocyte populations isolated from control and at various time periods (1, 2, 4, 12, and 24 hours) after surgical manipulation. Each value represents mean ± SD of 4 separate experiments. *Asterisk, P < .01 when compared with sham control; dagger, P < .01 when compared with preoperative state 1 hour after surgical manipulation.
Fig 2. Actin reorganization expressed as monomeric actin G/monomeric actin G + filamentous actin F (A) and thymidine incorporation expressed as disintegrations per minute (dpm)/10^7 cells (B) of villus, middle, and crypt enterocytes from control and various time periods after intestinal manipulation. Each value represents mean ± SD of 4 separate experiments. Asterisk, P < .01 when compared with sham control; dagger, P < .01 when compared with preoperative state 1 hour after surgical manipulation.

Statistical analysis. Data are expressed as mean ± SD. Statistical analysis was performed by using the Student t test.

RESULTS

The turnover of intestinal epithelial cells is a finely regulated process extending from undifferentiated crypt stem cells to terminally differentiated villus cells. Enterocytes were isolated to determine the susceptibility of these different cell populations at various stages of differentiation to surgical manipulation. They were grouped as villus, middle, and crypt fractions, and their viability was assessed by trypan blue exclusion. As can be seen...
from Fig 1, A, 1 hour after mechanical manipulation of the gut, a significant decrease in viability occurred in the crypt cells, which returned to control level by 24 hours after manipulation. The middle cells also showed a decrease in viability 1 hour after gut manipulation, whereas villus cell viability was unaffected.

The equilibrium between cell loss and cell production is essential for mucosal integrity. A time course study in the experimental rats showed a marked drop in the cell yield in crypt fraction 1 hour after intestinal manipulation as compared with sham control (Fig 1, B). This was associated with an increase in cell yield, both in middle and villus fraction. By 12 hours after surgical manipulation, the cell yield in different fractions was similar to the control level.

Cytoskeleton of the cell plays a crucial role in maintaining cellular integrity, morphology, and structure. Fig 2, A, shows the ratio of G/G+F (monomeric actin/monomeric actin + filamentous actin) in various cell populations from control levels and at different time periods after intestinal manipulation. It was observed that the G/G+F ratio remained unchanged in the villus cells, and the middle cells showed an increase in ratio with increasing time after manipulation. Actin reorganization was also evident in the crypt cells, where the G/G+F ratio was altered. These changes also returned to normal by 24 hours.

Changes in cell yield in different fractions and the actin reorganization after the surgical procedure probably indicated altered movement of proliferating cells. Cell proliferation was then assessed by thymidine incorporation by using the various cell fractions. Thymidine incorporation was significantly decreased in crypt cells 1 hour after experimentation as compared with the sham control (Fig 2, B). This decrease reversed with time and returned to control level by 24 hours. Middle cells showed marked increase in incorporation 4 hours after intestinal manipulation as compared with control, and by 24 hours, these cells reached the control level. No significant change in thymidine incorporation was seen in the villus cells. As expected, the proliferating crypt cells showed 2 to 3 times more thymidine incorporation as compared with middle and villus cells.

Alkaline phosphatase, a marker enzyme of cell differentiation in the intestinal mucosa, was assayed at different time periods after surgical manipulation (Fig 3, A). It was observed that specific activity of alkaline phosphatase decreased after 1 hour in all cell fractions and returned to control pattern within a few hours.

Superoxide generation by XO requires xanthine or hypoxanthine as a substrate. Adenosine deaminase (ADA) plays a role in nucleotide and purine metabolism, and its activity was measured in different cell populations of enterocytes after mechanical manipulation of the gut. As shown in Fig 3, B, ADA activity decreased significantly in the villus and middle cells after 1 hour, whereas an increase in activity was seen in crypt cells during the same period. This alteration in activity returned to control level in all the cell populations by 2 hours.

Mitochondria are an important source as well as target of free radicals. Studies on mitochondria from various cell populations were carried out to assess the functional changes that occur as a result of intestinal manipulation at different time periods. Oxygen uptake studies using mitochondria prepared from various cell populations showed decreased respiratory control ratio (RCR) in middle and crypt cells after 1 hour, and the ratio was normalized by 24 hours (Fig 4, A). Villus mitochondria showed an increase in RCR with time. Uncoupling of mitochondrial respiration is known to generate superoxide, and to assess this, an MTT reduction assay was performed. It was observed that mitochondria from all the cell fractions showed an increased MTT reduction after 1 hour, which reversed to control level by 24 hours (Fig 4, B). Damage to mitochondria results in swelling, and mechanical manipulation of the gut induced a significant increase in swelling in crypt and middle cell mitochondria after 1 hour, which returned to the normal level by 24 hours (Fig 5). Villus cell mitochondria also showed an increased swelling at 4 and 12 hours, which also returned to the control level by 24 hours.

DISCUSSION

The intestinal epithelium is a self-renewing monolayer arising from stem cells located at or near the base of the crypts. Our earlier study using a rat model showed that laparotomy with mild intestinal handling can result in permeability alterations and oxidative stress in the enterocytes, mainly as a result of activation of XO in the epithelium, and the damage to the epithelium is reversible with time. Rapid migration and proliferation of intestinal epithelial cells after different forms of intestinal injury have been identified as playing a key role in wound healing.

Studies on cell viability of enterocytes isolated by the chelation method from crypt, middle, and villus regions indicate that the crypt stem cells bear the brunt of damage induced by mechanical manipulation of the gut, which is reversible with time. Although the Weiser’s method used for the isolation of enterocyte population yields good sep-
The decrease in cell number in the crypt region, with a compensatory increase in middle and villus regions, probably reflects changes in cell migration patterns after gut manipulation. The cellular turnover is essential to prevent uncontrolled growth. Surgical manipulation affects cell numbers in various populations, a fact reflected in the altered cell yield.

Fig 3. Alkaline phosphatase (A) and adenosine deaminase (B) activity in different cell populations of isolated enterocytes from control and various time periods after surgical manipulation. All assays were carried out as described in the “Methods.” Each value represents mean ± SD of 6 separate estimations. Asterisk, \( P < .01 \) when compared with control; dagger, \( P < .01 \) when compared with preoperative state 1 hour after surgical manipulation.
The cytoskeleton plays an important role in cell migration in the intestine, and actin is an important component of the cytoskeleton. Actin, being a principal protein in the cell cortex, plays a central role in maintaining the cellular integrity, morphology, and the structure of the cytoplasmic matrix. The importance of the complex network of actin cytoskeleton in the maintenance of normal cellular homeostasis and barrier function has been shown recently. Surgical manipulation was found to induce dynamic changes in the state of actin polymerization, reflected in the change in G/G+F actin ratios. These changes were especially prominent in the crypt and middle regions, whereas the villus cells were unaffected. This probably indicates actin reorganization in the crypt and middle fractions, which would explain the altered cell yield, since it has been shown that the migrating cells undergo alterations in the actin cytoskeletal protein. Albers et al have shown that in vitro repair of the

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**Fig 4.** Respiratory control ratio (A) and MTT reduction (B) of enterocyte mitochondria of different cell populations of enterocytes from control and various time periods after surgical manipulation. Each value represents mean ± SD of 4 separate experiments. Assays were carried out as described in “Methods.” Asterisk, $P < .01$ when compared with control. Dagger, $P < .01$ when compared with preoperative state 1 hour after surgical manipulation.
native small intestinal epithelium is functionally and structurally dependent on major changes in the cytoskeleton of cells involved in re-establishing the epithelial monolayer over a complex extracellular matrix. Our earlier study demonstrated oxidative stress in the intestine after surgical manipulation, and the actin cytoskeleton has been shown to play an important role in the recovery of cell adhesion after oxidative stress injury. Studies have also shown decreased crypt cell proliferation after thermal injury. After an injury, stem cells proliferate to increase their number and give rise to transit cells that proliferate to form regenerative crypts. Increased incorporation of thymidine seen 2 hours after surgical manipulation indicates that the stem cells at the base of the crypt proliferate to replenish the damaged cells, and the relative displacement of daughter cells is probably, in part, a result of subsequent divisions of the intervening cells. The movement of the cells from the crypt to villus was increased probably as a result of continuous increased proliferation in the crypt. This could partly explain the increased incorporation in the middle cells after 4 hours. Increased cell migration could possibly result in undifferentiated cells being pushed rapidly to the middle and villus regions. A marker enzyme for differentiation, alkaline phosphatase, was then measured, but no change in enzyme distribution was evident. However, surgical manipulation induced a decrease in ALP activity by 1 hour in all the fractions, and this is again indicative of oxidative stress-induced damage, since this enzyme is susceptible to oxygen-free radicals. Earlier work has indicated activation of XO during mechanical manipulation of the small bowel, and this is the main source of free radicals in the cell. This enzyme activity is undetectable normally in the crypt cells but appears after intestinal manipulation. XO generates free radicals during the conversion of xanthine and hypoxanthine to uric acid. Inosine is a precursor of hypoxanthine and xanthine and is generated by the action of ADA on adenosine. To determine whether substrate availability had a role in XO-induced damage, we also measured ADA activity in various cell populations after manipulation of the gut. ADA activity was decreased in villus and middle cells and increased in crypt cells after 1 hour, which returned to control level by 24 hours. This suggests that increased activity of adenosine deaminase might provide the substrate for free radical generation in the crypt cells. It has been previously shown that inosine triphosphate, the deamination product of ATP, is one of the superoxide-generating components of clastogenic plasma factors in scleroderma patients.

Maintaining a balance between cell production and cell loss is a constant process in the intestinal epithelium. An increase in cell proliferation could also lead to alteration in cell movement, illustrated by the actin polymerization. It has been shown that mechanical manipulation of the gut can affect cell proliferation, and tritiated thymidine incorporation studies have demonstrated a decreased crypt cell proliferation after 1 hour. Studies have also shown decreased crypt cell proliferation after thermal injury. After an injury, stem cells proliferate to increase their number and give rise to transit cells that proliferate to form regenerative crypts. Increased incorporation of thymidine seen 2 hours after surgical manipulation indicates that the stem cells at the base of the crypt proliferate to replenish the damaged cells, and the relative displacement of daughter cells is probably, in part, a result of subsequent divisions of the intervening cells. The movement of the cells from the crypt to villus was increased probably as a result of continuous increased proliferation in the crypt. This could partly explain the increased incorporation in the middle cells after 4 hours. Increased cell migration could possibly result in undifferentiated cells being pushed rapidly to the middle and villus regions. A marker enzyme for differentiation, alkaline phosphatase, was then measured, but no change in enzyme distribution was evident. However, surgical manipulation induced a decrease in ALP activity by 1 hour in all the fractions, and this is again indicative of oxidative stress-induced damage, since this enzyme is susceptible to oxygen-free radicals. Earlier work has indicated activation of XO during mechanical manipulation of the small bowel, and this is the main source of free radicals in the cell. This enzyme activity is undetectable normally in the crypt cells but appears after intestinal manipulation. XO generates free radicals during the conversion of xanthine and hypoxanthine to uric acid. Inosine is a precursor of hypoxanthine and xanthine and is generated by the action of ADA on adenosine. To determine whether substrate availability had a role in XO-induced damage, we also measured ADA activity in various cell populations after manipulation of the gut. ADA activity was decreased in villus and middle cells and increased in crypt cells after 1 hour, which returned to control level by 24 hours. This suggests that increased activity of adenosine deaminase might provide the substrate for free radical generation in the crypt cells. It has been previously shown that inosine triphosphate, the deamination product of ATP, is one of the superoxide-generating components of clastogenic plasma factors in scleroderma patients.

Normal mitochondrial function is essential for cell survival, and a prominent feature of cell dam-
age caused by oxidative stress is damage to the mitochondria. To confirm the cell damage at the subcellular level, studies using mitochondria prepared from villus and crypt cells were carried out. Mitochondrial studies also have shown maximum damage 1 hour after gut manipulation. Measurement of RCR indicated uncoupling of mitochondria in crypt and middle cells. This uncoupling of mitochondria could contribute to generation of oxygen-free radicals and functional alterations in the mitochondria. MTT reduction assay confirmed the generation of superoxide anions. Oxidants can also induce swelling of the mitochondria, and this was also seen more prominently in the crypt and middle cells. All these changes were seen 1 hour after mechanical manipulation of the gut, which returned to control pattern within 24 hours.

In conclusion, this study has shown that surgical manipulation of the intestine induces 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 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. An understanding of this intricate balance of cell populations in the intestinal mucosa will probably help researchers to delineate the mechanism of susceptibility and reversibility of this damage.

REFERENCES