Effects of reactive oxygen species on mitochondrial content and integrity of human anastomotic colorectal dehiscence: A preliminary DNA study


BACKGROUND: Anastomotic dehiscence is one of the most severe complications of colorectal surgery. Gaining insight into the molecular mechanisms responsible for the development of anastomotic dehiscence following colorectal surgery is important for the reduction of postoperative complications.

OBJECTIVE: Based on the close relationship between surgical stress and oxidative stress, the present study aimed to determine whether a correlation exists between increased levels of reactive oxygen species and colorectal anastomotic dehiscence.

METHODS: Patients who underwent surgical resection for colorectal cancer were divided into three groups: patients with anastomotic dehiscence (group 1); patients without dehiscence who underwent neoadjuvant radiochemotherapy (group 2); and patients without anastomotic dehiscence who did not undergo neoadjuvant radiochemotherapy (group 3). Quantitative polymerase chain reaction and real-time polymerase chain reaction assays were performed to measure nuclear DNA and mitochondrial DNA (mtDNA) content, and possible oxidative damage to nonmalignant colon and rectal tissues adjacent to the anastomoses.

RESULTS: mtDNA content was reduced in the colon tissue of patients in groups 1 and 2. Rectal mtDNA was found to be more damaged than colonic mtDNAs in all groups. The 4977 bp common deletion was observed in the mtDNA of tissues from both the colon and rectum of all patients.

DISCUSSION: Patients in groups 1 and 2 were more similar to one another than to group 3, probably due to higher levels of reactive oxygen species in the mitochondria; the greater damage found in the rectum suggests that dehiscence originates primarily from the rectal area.

CONCLUSIONS: The present study of mtDNA analyses of normal human colon and rectal tissues from patients with colorectal cancer is among the first of its kind.

Key Words: Anastomotic dehiscence; Mitochondrial DNA; Oxidative DNA damage; Reactive oxygen species

It is well known that surgical stress is closely related to oxidative stress, with consequent formation of molecules acting at the systemic level, modifying the main physiological activities of different tissues. Several studies have demonstrated that laparotomy and intestinal manipulation cause a temporary decrease of xanthine-oxidase enterocyte activity and catalase activity (1,2). This phenomenon, associated with the uncoupling of mitochondrial oxidative phosphorylation, leads to superoxide and peroxide generation (3). Superoxide formed as a result of surgical stress can activate cytosolic and mitochondrial proteases in intestinal epithelial cells that stimulate lipid peroxidation and cascade damage primarily involving the membranes (4). Tissue repair mechanisms are negatively influenced by the presence of reactive oxygen species (ROS), which probably also contribute to colorectal anastomotic dehiscence. To date, however, very little is known about the molecular mechanisms underlying colorectal anastomotic dehiscence in humans.

Anastomotic dehiscence is one of the most severe complications of colorectal surgery, leading to infection, sepsis, and increased morbidity

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and mortality, and adversely affecting the length of hospitalization, costs and cancer recurrence. Notwithstanding the improvement in surgical techniques, the rate of anastomotic dehiscence remains high – from 3% to 15% (5-9) – mainly occurring after rectal resection. Introduction of neoadjuvant chemotherapy and radiotherapy targeting the rectum and pelvis may be a factor that leads to an increase in anastomotic dehiscence. Many factors seem to play a pivotal role in the anastomosis healing process including suitable tissue perfusion, innovative anastomotic surgical techniques and the experience of the operating surgeon (5,10,11). However, the pathogenesis and the causes of this phenomenon, which generally arises five to seven days after surgery, remain unclear. In recent years, scientists have shifted their focus from clinical or technical factors related to the process of tissue repair, to possible pathogenetic biochemical and/or ultrastructural mechanisms. For several years, collagen synthesis and related disorders have been considered to be important factors able to influence anastomosis strength (12-14). The hypothesis that collagen defects are linked to the development of anastomotic dehiscence is based on experimental evidence from patients in whom abdominal wall hernias are often associated with the presence of connective tissue disorders (15,16).

Similarly, it can be assumed that colorectal anastomotic dehiscence may be connected to a collagen metabolism defect such as a lowered collagen type I/III ratio or an increase in the expression of matrix metalloproteinases (17,18) – a family of enzymes that degrade extracellular matrix components. In previous studies, it has been shown that the breaking strength of colonic anastomosis could be enhanced by substances that have increased collagen content such as a biosynthetic growth hormone (19-22), gentamicin (23) or matrix metalloproteinase (24-27).

The present investigation focused on the relationship between increased levels of oxidative stress and colorectal anastomotic complications. The molecular mechanisms responsible for this phenomenon have yet to be investigated – the current study aimed to take the first step toward gaining a better understanding of such mechanisms.

Because ROS trigger the oxidation of macromolecules, such as proteins, lipids and nucleic acids, we evaluated their possible oxidative effects on mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) in three groups of patients with colorectal cancer: those with anastomotic dehiscence; those without dehiscence who underwent neoadjuvant radiochemotherapy; and those without dehiscence who did not undergo neoadjuvant radiochemotherapy.

Analyses were performed on normal human colon and rectal tissues that were sutured in colorectal anastomosis procedures performed on the previously mentioned patients. nDNA and mtDNA content and possible oxidative damage were analyzed by quantitative polymerase chain reaction (PCR) and real-time PCR.

Moreover, radiotherapy induces oxidative stress that repair systems cannot efficiently counteract, thereby leading to a probable tendency toward anastomotic dehiscence. Many previous experimental trials in animals (28-31) – especially rats – evaluated oxidative stress levels in the colon; however, these investigations did not reproduce real human conditions with respect to radiotherapy doses nor with respect to surgical technique. We also evaluated the association between tissue damage due to neoadjuvant treatments (i.e., radiochemotherapy) and anastomotic outcome.

### METHODS

**Patients**

Thirty-four patients with rectal cancer who underwent anterior rectal resection at the Hospital of University ‘Campus Bio-Medico’ of Rome (Italy) between 2006 and 2009 were selected. All patients provided written informed consent. The present study was performed in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and was approved ethically by the Ethics Committee of University Campus Bio-Medico (reference number 03/08 protocol INT ComEt CBM).

Patients with connective tissue pathologies, microangiopathies, ulcerative colitis or Crohn’s disease, or vasculitis were excluded.

Seventeen men and 17 women with a mean age of 68.2 years (range 48 to 93 years) were divided into three groups: those who did not undergo neoadjuvant therapy and developed dehiscence (group 1 \( n=8 \)); those who received neoadjuvant therapy but did not develop dehiscence (group 2 \( n=10 \)); and those who did not receive neoadjuvant therapy and did not develop dehiscence (group 3 \( n=16 \)) (Table 1). Patients who received preoperative treatment were affected with lower rectal cancer (stage I or II); this treatment is routinely performed at the institution to improve postoperative outcome.

**Necadjuvant therapy** consisted of variable and personalized chemotherapy, and a standard radiotherapy of 45 gray (Gy) fractionated as 180 cGy/die directed to the rectum and lymph drainage, followed by 5.4 Gy (total dose) fractionated as 180 cGy/die directed to the rectal lesion and to the mesorectum. The interval of time between radiotherapy and surgical resection was approximately one to two months. The experimental protocol was approved by the ethics committee.

**Isolation of total DNA**

Two tissue segments (one from the colon and one from the rectum) measuring 4 mm × 5 mm were taken from the edges of the resected specimens. Total DNA was isolated using the QIAmp DNA mini kit (Qiagen, USA) according to the manufacturer’s instructions. Total tissue DNA concentration was spectrophotometrically determined at 260 nm using a Beckman DU-640 spectrophotometer (Beckman, USA).

**Long PCR and quantitative PCR**

Long PCR was performed using a GeneAmp PCR System 9700 thermocycler (PE Applied Biosystems, USA). The reaction mixture contained 50 ng or 100 ng of total template DNA (to amplify mtDNA and nuclear DNA [nDNA], respectively), 2.5 μL of 10X buffer, 200 μM dNTPs, 0.5 μM of each primer and 0.5 μL of Advantage-2-polymerase (Clonotech, USA) in a final volume of 25 μL. Primers Af-Ar were used to amplify a 16.2 kb fragment of mtDNA, and primers Bf-Br were used to amplify a 13.5 kb fragment of nDNA (Table 2). The PCR parameters are reported in Table 3. The long PCR amplification products were electrophoresed on a 0.8% agarose Tris/Borate/EDTA gel, stained with ethidium bromide (0.3 μg/mL) and quantified by densitometric analysis of the intensity of bands using Quantity One software version 4.01 (Bio-Rad, USA).

**Detection of the mitochondrial 4977 bp common deletion**

To assess the presence of the common deletion, which involves nucleotides from position 8470 to 13447 (accession number NC_001807.4), a pair of primers were used: F2 (Table 2) located at nucleotide position 8283 of the complete Homo sapiens mitochondrial sequence (accession number NC_001807.4) and R2 (Table 2) located at nucleotide position 13610 (NC_001807.4). These primers produce a 4347 bp amplicon from intact mtDNA and an amplicon of 370 bp in the presence of a deletion (5347 to 4977) (Figure 1). PCR was performed in a final volume of 25 μL containing 50 ng of genomic DNA, 0.5 μM of each of the above primers, 200 μM dNTPs, 2.5 μL of 10X buffer, 1.5 mM MgCl2 and 1.5 units of Taq DNA polymerase (Diathema, Italy). Reaction conditions were as follows: denaturation at 95°C for 10 min, followed by 30 cycles of 94°C for 30 s, 59°C for 30 s

<table>
<thead>
<tr>
<th>Group</th>
<th>Patients, n</th>
<th>Dehiscence</th>
<th>RT-CH</th>
<th>Sex, Male/female, n</th>
<th>Mean age, years</th>
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<tr>
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<td>16</td>
<td>No</td>
<td>No</td>
<td>6/10</td>
<td>64.1</td>
</tr>
</tbody>
</table>

**RT-CH Neoadjuvant therapy**
and 72°C for 30 s, with final extension at 72°C for 7 min. The identities of the amplified products were confirmed by sequencing the products purified from the gel and comparing them with the appropriate mtDNA sequence (Figure 1).

Real-time PCR

Real-time PCR was performed to determine the ratio of mtDNA to nDNA, and to quantify ND4 lesions by amplifying both ND1 and ND4 (NADH dehydrogenase complex 1 and 4, respectively) as mtDNA and β-actin as nDNA. The first ratio was calculated as ND1/β-actin and ND4/β-actin. ND4 lesion frequency has been determined to correlate with the quantity of amplified ND1 and ND4.

Real-time quantitative PCR (QPCR) was performed using an iCycler iQ Multi-Color Real-time PCR Detection System (Bio-Rad, USA) and 2x Quantitect SYBR Green PCR kit (Qiagen, USA). The reaction mix (25 μL final volume) consisted of the following: 12.5 μL Mix Hot-Start (Qiagen, USA), 50 ng/μL template DNA, 2 μL SYBR Green and 0.3 μM of each primer (Table 2). The PCR conditions were as follows: hot start at 95°C for 10 min, followed by 40 cycles of the two steps at 95°C for 30 s and 60°C for 30 s. The threshold cycle was determined on the linear phase of PCRs using the iCycler iQ Optical System Software version 3 (BioRad, USA). The specificity of the amplification products was confirmed by examining thermal denaturation plots and by sample separation on a 3% agarose gel. ND1 and β-actin gene copy numbers were determined by interpolating the threshold cycle from standard curves, which were obtained using DNA from the human keratinocyte cell line NCTC 2544 (Figure 2). The ratios were obtained relating these mtDNA and nDNA quantities. Samples were tested in triplicate for each gene.

Statistical analysis

Statistical analysis was performed using Statistica version 6.1 (Statsoft, Italy). The variables among the groups were compared using a two-way ANOVA, and the Bonferroni test was used post hoc. Differences among the groups were considered to be statistically significant at P<0.05, with a confidence level of 95%.

RESULTS

Oxidative damage was evaluated in nDNA and mtDNA. Total genomic DNA was extracted from fragments of colon and rectum obtained from the margin of resected specimens, spectrophotometrically quantified and analyzed on an agarose gel to confirm its size and integrity (data not shown).

Two fragments of nDNA and mtDNA, corresponding to the long PCR products were quantified by densitometric analysis after gel electrophoresis (QPCR).

Polymerization activity of any thermostable polymerase is terminated in the presence of DNA lesions, such as strand breaks, abasic sites and some base modifications (eg, 8oxodA). Therefore, if DNA damage was severe, the high number of lesions on the template would induce a lower yield of amplification products (32).

The QPCR assay had already been established with these primers in the authors’ laboratory (33,34). The QPCR analyses of the two genomes showed that all three groups were not significantly different from one another, suggesting that oxidative damage in mtDNA (Figure 3A) or in nDNA was not extensive (Figure 3B).
To evaluate mtDNA content in colon and rectum tissues, real-time PCR of mitochondrial ND1 and ND4 genes normalized with the nuclear β-actin gene was performed. Results showed that there were no significant differences in either ND1/β-actin or ND4/β-actin ratios in the rectal tissue of all three groups of patients (Figure 4). In contrast, a statistically significant greater amount of mtDNA with ND1/β-actin was found in the colon of group 3 patients than in the other two groups. ND4/β-actin analysis showed no differences among the three groups, while it showed a significant difference between the colon and rectum of group 3 patients (Figure 4B). Differences between the ND4/β-actin results and those of ND1/β-actin led to the search for the 4977 bp common deletion, which includes the ND4 gene. Therefore, PCR using primers F2-R2 that amplified a product of 370 bp when the common deletion is present was performed. All patients were positive for this mutation (data not shown) both in rectal and colonic tissues; this evidence was confirmed and quantified by real-time QPCR reflected by the ND1/ND4 ratio. This final analysis (Figure 5) highlighted that rectal tissue was more damaged than colon tissue in each group, and that the differences were statistically significant.

DISCUSSION

Anastomotic dehiscence represents one of the most frequent complications of colorectal surgery. The introduction of important technical innovations (new suture threads, mechanic suturing apparatus and mini-invasive operating techniques) has improved standardisation in the various roles of this organelle including energy production, electron transport, cell motility, cell proliferation and apoptosis (41-43). Alteration of mitochondrial function has been discovered in many tumours (44,45) as well as punctiform mutations or deletions in dislocation loops or in coding regions, and little deletion of mitochondrial DNA (mtDNA) in tumours of the colon (46-48), breast (49), stomach (50), kidney (51), esophagus (52), thyroid (53), head and neck (54). There is also evidence that mtDNA alterations can influence cell susceptibility to therapeutic cancer-fighting agents (42,55).

mtDNA is a double-stranded molecule 16,569 bp long, and was completely sequenced in 1981 (35). This gene encodes 13 polypeptides, all of which are essential in electron transport and ATP formation (36); it is 10 times more susceptible to insults than nDNA (37). This condition is easily attributable to various factors such as exposure to high levels of ROS produced during oxidative phosphorylation (38), the absence of histone protection and limited DNA repair systems (39); in the mitochondria, a base excision repair system has been documented more extensively than the nucleotide excision repair system (40). Consequently, oxidative stress damage to mtDNA can lead to incorrect gene expression, with a consequent loss of membrane potential and a vicious circle input between ROS generation and mtDNA oxidation owing to the complete lack of ATP synthesis and, finally, cell death.

Recently, scientists have focused on the mitochondrion because of the various roles this organelle including energy production, electron transport, cell motility, cell proliferation and apoptosis (41-43). Alteration of mitochondrial function has been discovered in many tumours (44,45) as well as punctiform mutations or deletions in dislocation loops or in coding regions, and little deletion of mitochondrial DNA in tumours of the colon (46-48), breast (49), stomach (50), kidney (51), esophagus (52), thyroid (53), head and neck (54). There is also evidence that mtDNA alterations can influence cell susceptibility to therapeutic cancer-fighting agents (42,55).

Results obtained with QPCR did not show significant differences among the three groups of patients, suggesting that oxidative injury is
among the three groups of patients. The colons of patients in group 3, particular, there were no significant differences in rectum analyses aged than the colon in that ND1 was greater than ND4 (Figure 5). In contrast, the same assays of colon tissue revealed differences samples in which changes in rectum mtDNA content were expected. Results obtained by quantitative real-time PCR were more interesting Colon and rectum mtDNA content were evaluated by ND1/β-actin and ND4/β-actin ratios. These assays highlighted no differences in rectum samples among the three groups, or in neoadjuvant treated samples in which changes in rectum mtDNA content were expected. In contrast, the same assays of colon tissue revealed differences between group 3 and the other two groups, which proved to be more similar to one another. The significant reduction in colon mtDNA content in group 2 (Figure 4A) when compared with patients in group 3 could be attributable to chemotherapy; however, the reason for the reduction of mtDNA content in group 1 remains unclear. Because radiochemotherapy increases ROS production (28-31), the results of the present study may lend support to the initial hypothesis in which there is increased ROS production; the greater damage that we found in the rectum suggests that dehiscence may originate primarily from the rectal side; hence, future research should focus on this tissue using transcriptomic analyses to detect differentially expressed genes as possible markers of dehiscence. Despite the small sample size examined in the present study, we offer the following conclusions: groups 1 and 2 were found to be more similar with one another than group 3, a finding that might support the initial hypothesis in which there is increased ROS production; the greater damage that we found in the rectum suggests that dehiscence may originate primarily from the rectal side; hence, future research should focus on this tissue using transcriptomic analyses to detect differentially expressed genes as possible markers of dehiscence.

**REFERENCES**


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