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ORIGINAL ARTICLE

Barbara E. Wildhaber · Hua Yang
Arnold G. Coran · Daniel H. Teitelbaum

Gene alteration of intestinal intraepithelial lymphocytes in response to massive small bowel resection

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Abstract ***Background***: The intestinal adaptive response [increased epithelial cell (EC) proliferation and apoptosis] after massive small bowel resection (SBR) is partially controlled by intraepithelial lymphocytes (IEL). To identify IEL factors contributing to EC adaptation post-SBR we utilized microarray assays. ***Methods***: Mice underwent a 70% SBR (SBR1w/SBR4w) or sham operation (Sham1w/Sham4w). After 1 or 4 weeks (1w, 4w) small bowel was harvested, and IEL isolated. Determination of the EC-proliferation rate used BrdU incorporation, and of the EC-apoptotic rate used Annexin V staining. Affymetrix system microarrays (12,491 genes) were performed to examine IEL-mRNA expression. Results were considered significant if fold-change (FC) between groups was >2 and $P<0.05$ (F-test), or $FC>3$ and $0.05>P>0.01$, or $FC>4$ and $P>0.05$. Significant genes were confirmed by conventional RT-PCR. ***Results***: The SBR EC-proliferation rate increased significantly in both 1w and 4w groups compared to Sham: SBR1w 0.24 ± 0.07 vs. Sham1w 0.12 ± 0.02 ($P=0.03$); SBR4w 0.35 ± 0.04 vs. Sham4w 0.19 ± 0.02 ($P<0.01$). The EC-apoptotic rate was unchanged in the 1w group, but significantly differed from controls after 4 weeks: SBR4w 39.92 ± 6.78 vs. Sham4w 12.56 ± 6.44 ($P<0.01$). Microarray results were analyzed to identify potential growth-modifying IEL genes. The following were identified (function in parenthesis; A, apoptosis; P, proliferation): lipocalin 2 (promotes A), angiotensin converting enzyme (increases A), Rap2 interacting protein (reduces A, promotes P), amphiregulin (promotes P) and leucine-rich-α2-glycoprotein (promotes A, reduces P). Based on RT-PCR results these genes showed significant changes between groups. The increase in ACE at 1w preceded the observed apoptotic changes. The alterations in lipocalin 2, Rap2 and amphiregulin at 4w coincided with the marked changes in growth and apoptosis in the SBR mice. ***Conclusions***: IEL undergo temporal changes after SBR. These findings provide profound insight into potential IEL-dependent regulation of EC homeostasis post-SBR.

Keywords Short bowel syndrome · Intraepithelial lymphocytes · Apoptosis · Proliferation · Microarray

B. E. Wildhaber · H. Yang · A. G. Coran · D. H. Teitelbaum (✉)
Department of Surgery, Section of Pediatric Surgery,
C.S. Mott Children's Hospital, University of Michigan,
Mott F3970, Box 0245, Ann Arbor, Michigan, 48109, USA
E-mail: dttlbm@umich.edu
Tel.: +734-764-4151
Fax: +734-936-9784

Introduction

Massive small bowel resection (SBR) leads to an adaptive response in the residual intestine. Following SBR the remaining intestine undergoes important changes to compensate for the loss of mucosal surface area, whereby the epithelial cell (EC)-proliferation and -apoptosis rates are markedly altered. After SBR, the equilibrium of EC-proliferation and -apoptosis, which under normal physiologic circumstances is balanced, alters in favor of compensatory mucosal growth.

Mechanisms and mediators of intestinal adaptation are known to be multifactorial. One of the mechanisms of intestinal growth is thought to include the action of intraepithelial lymphocytes (IEL). IEL secrete a variety of cytokines and growth factors, like KGF, IFN-γ, TNF-α or IL-12, that play an important role in sustaining EC growth and structure [1, 2, 3]. IEL may directly influence the regulation of the intestinal turnover of the EC. There is reason to believe that IEL express even more mediators than those presently known. To identify IEL factors contributing to EC adaptation after SBR, we utilized microarray assays, which allow simultaneous expression of thousands of genes. This study focused on the expression of growth-related genes, which might provide increased insight into IEL function in small bowel adaptation after massive SBR.

Materials and methods

Animals

Male, 2 month old, C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were initially fed *ad libitum* with normal mouse chow and water for an acclimation period of 7 days; 24 h before surgery chow was exchanged to micro-stabilized rodent liquid diet (Test-Diet, Richmond, IN). Mice were divided into four groups ($n = 6$), and operations were performed. Anesthesia was achieved using sodium pentobarbital (50 mg/kg body weight, intraperitoneally). Massive small bowel resection (SBR1w/4w): Through an abdominal midline incision, 70% of the small bowel was resected (i.e., bowel between 3.5 cm distal to the ligament of Treitz and 3.5 cm proximal to the ileocecal valve), followed by and an end-to-end jejunoileal anastomosis with 7/0 monofilament, resorbable suture [4]. Postoperatively, a subcutaneous bolus of 3 ml saline was given to maintain intravascular hydration status. For the Sham operation (Sham1w/4w), the intestine was transsected and re-anastomosed without bowel resection.

Postoperatively, *ad libitum* water and liquid diet were reintroduced. If mice were not sacrificed after 1 week (w), food was changed to standard chow. The small intestine was allowed to adapt for 1w (SBR1w, Sham1w) or 4w (SBR4w, Sham4w) to assess temporal changes. Animals were killed using CO_2, and the intestine was harvested.

Mucosal cell isolation

Isolation of mucosal cells was performed using a slightly modified previously described protocol [5]. Mucosal epithelium was harvested from segments of intestine with an extraction buffer (l mM EDTA, l mM dithiotheritol in phosphate buffer saline) and centrifuged. In order to extract EC, the centrifuged pellets were purified in 20% isotonic Percoll (Pharmacia, Piscataway, NJ). Viability exceeded 95% using trypan blue exclusion staining. The cell suspension contained a purified mixture of EC and IEL at a ratio of 70:30.

IEL purification

Magnetic beads conjugated with antibody to CD45 (lymphocyte-specific) were used to remove epithelial cells (BioMag SelectaPure Anti-Mouse CD 45R Antibody Particles, Polyscience Inc., Warrington, PA). Cells bound to beads were considered purified IEL, and the supernatant contained EC. Flow cytometry confirmed purity of sorted cells, which was >99%.

Microarray assay

Total RNA was isolated using Trizol (Gibco BRL, Gaithersburg, MD) and was purified with the RNeasy Mini Kit (Qiagen, Valencia, CA). Affymetrix system microarray chips (Affymetrix, Inc., Santa Clara, CA) allowed simultaneous interrogation of 12,491 full-length mouse genes and EST clusters from the UniGene database. Hybridization and analysis were performed by the University of Michigan NIDDK Biotechnology Center. Probe-pair measures were obtained from the images of each chip using Microarray Suite 4.0 software (Affymetrix).

Reverse transcriptase polymerase chain reaction (RT-PCR)

IEL mRNA (poly-A positive) was reversed transcribed into cDNA following a standard protocol [5]. Specific primers for selected gene sequences were designed (Table 1). PCR and gel were run under standard conditions [5]. To ensure that DNA product was generated at the exponential portion of the product curve, the following cycle numbers were used: 28 cycles for ACE, 32 cycles for Rap2 interacting protein and 34 cycles for lipocalin 2, amphiregulin and leucine-rich-α2-glycoprotein. Gel bands were analyzed by DNA sequencing technology (University of Michigan's Sequencing Core) to ensure the correct product. Kodak EDAS System (Rochester, NY) was used for imaging and quantification. Results were expressed as the ratio of the investigated mRNA over the β-actin mRNA expression.

Epithelial cell proliferation assay

Mice were injected intraperitoneally with 5-bromodeoxyuridine (BrdU, 50 mg/kg body weight, Roche Diagnostic Corporation, IN) 24 h before harvest. Immunohistochemical staining was performed using the BrdU In-Situ detection Kit (BD PharMingen, San Diego, CA). Proliferation rate is expressed as the ratio of BrdU positive cells over the total number of epithelial cells of a crypt-villus complex. Each assessment of proliferation consists of the mean of 14 different high power microscopic fields.

Flow cytometry

Apoptosis was determined by flow cytometry based on the cell surface expression of phosphatidylserine (Annexin V). Annexin V assay was performed with the Apoptosis Kit from BD PharMingen (San Diego, CA). Staining with propidium iodide was used to monitor cell necrosis. The apoptotic rate was determined by gating on EC and detecting the percent of Annexin V staining as percentage of total EC.

Statistical analysis

Significant alteration (SBS vs. Sham) of a gene on the microarray chip was defined as a fold-change (FC) >2 and P value <0.01 (F-test), or FC>3 and $0.05> P >0.01$, or FC>4 and $P >0.05$. Other results are expressed as mean $\pm$ standard deviation. These results were analyzed using the t-test, statistical significance equals $P <0.05$.

Table 1 Specific primer data. *bp* base pairs

Name (length of cDNA product)	GeneBank accession no.	Forward primer	Reverse primer
β-actin (194 bp)	NM_007393	gagggaaatcgtgcgtgacat	agaaggaaggctggaaaagag
Lipocalin 2 (206 bp)	XM_130171	ccagttcgccatggtatttt	cacactcaccacccattcag
Angiotensin converting enzyme (200 bp)	XM_126568	gcagacctggtccaacatct	catcggtgggcttctctaac
Rap2 interacting protein (203 bp)	NM_016759	ttcagctcagatggacaacg	gtagttcggttgtcccgaag
Amphiregulin (198 bp)	NM_009704	cggtggaaccaatgagaact	tttcgcttatggtggaaacc
Leucin-rich-α2-glycoprotein (203 bp)	NM_029796	tcccatgtrcagtgtgcagat	tcagcctaggagccgtttta

Results

EC proliferation

SBR1w vs. Sham1w showed a significant increase in the proliferation rate, which was further increased after 4w (see Table 1, Fig. 1).

EC apoptosis and necrosis

EC-apoptosis 1w postoperatively was increased in both groups (Sham1w, SBR1w) (Table 2, Fig. 2). However, after 4w the EC-apoptotic rate significantly differed between groups with a persistent elevation in SBR4w ($P > 0.05$ vs. SBR1w) and a marked decline in the Sham4w mice ($P = 0.01$ vs. Sham1w). Necrosis showed no significant changes between all groups.

Expression of mRNA

The Affymetrix microarray chip tested for 12,491 mouse genes (Table 3). After 1w 65 genes and after 4w 254 genes were significantly changed between SBS and sham groups, according to our criteria. These genes were analyzed for their function, and factors related to growth regulation were chosen for confirmation by RT-PCR. The following IEL genes were selected (function in parenthesis): lipocalin 2 (promotes apoptosis), angiotensin converting enzyme (ACE, promotes apoptosis), Rap2 interacting protein (reduces apoptosis, promotes proliferation), amphiregulin (promotes proliferation) and leucine-rich-α2-glycoprotein (promotes apoptosis, reduces proliferation). PCR results confirmed significant changes between groups. The significant increase in ACE 1w postoperatively (indicating increased apoptosis in SBR1w vs. Sham1w) and the increase in Rap2 interacting protein after 4w (indicating decreased apoptosis in SBR4w vs. SBR1w) preceded the observed apoptotic changes. The alterations in lipocalin 2 (indicating decreased apoptosis in Sham4w vs. Sham1w), amphiregulin (indicating increased proliferation in SBR4w vs. SBR1w) and leucine-rich-α2-glycoprotein (indicating increased apoptosis in SBR4w vs. Sham4w) coincided with the marked homeostatic variations.

Table 2 Changes in rate of epithelial cell (EC)-proliferation, -apoptosis and -necrosis rate 1 and 4 weeks postoperatively. Proliferation rate is expressed as the ratio of BrdU positive cells over the total number of EC of a crypt-villus complex. Apoptotic rate is detected by gating on EC with flow cytometry and detecting the percent of Annexin V staining as percentage of total EC. Note the significant change in proliferation between groups 1 and 4 weeks postoperatively. The apoptotic rate after 4 weeks showed a persistent elevation in SBR mice and a decline in the sham operated group. Necrosis showed no significant changes between groups. *$P < 0.05$ Sham1w vs. SBR1w, ** $P < 0.01$ Sham4w vs. SBR4w

Group	EC proliferation rate (positive/total)	EC apoptosis rate (% of EC population)	EC necrosis rate (% of EC population)
SBR1w	0.24 ± 0.07	36.0 ± 19.9	11.6 ± 7.1
Sham1w	0.12 ± 0.02*	36.4 ± 10.1	9.6 ± 4.6
SBR4w	0.35 ± 0.04	39.9 ± 6.8	14.6 ± 5.7
Sham4w	0.19 ± 0.02**	12.6 ± 6.4**	12.8 ± 9.3

Discussion

Epithelial cell (EC) homeostasis is maintained on a day-to-day basis by a fine balance between EC-proliferation and -apoptosis. The intestinal mucosa permanently

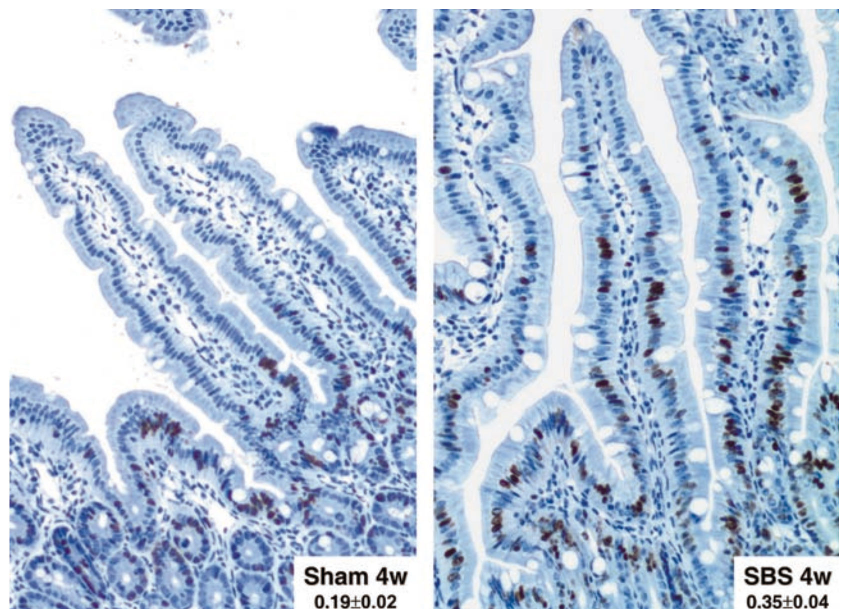

Fig. 1 BrdU staining of small bowel biopsies. Proliferation rate is expressed as the ratio of BrdU positive cells over the total number of epithelial cells (*EC*) of a crypt-villus complex. Note the markedly increased EC-proliferation rate (*dark stained cells*) after massive small bowel resection (*SBR4w*). Magnification ×160

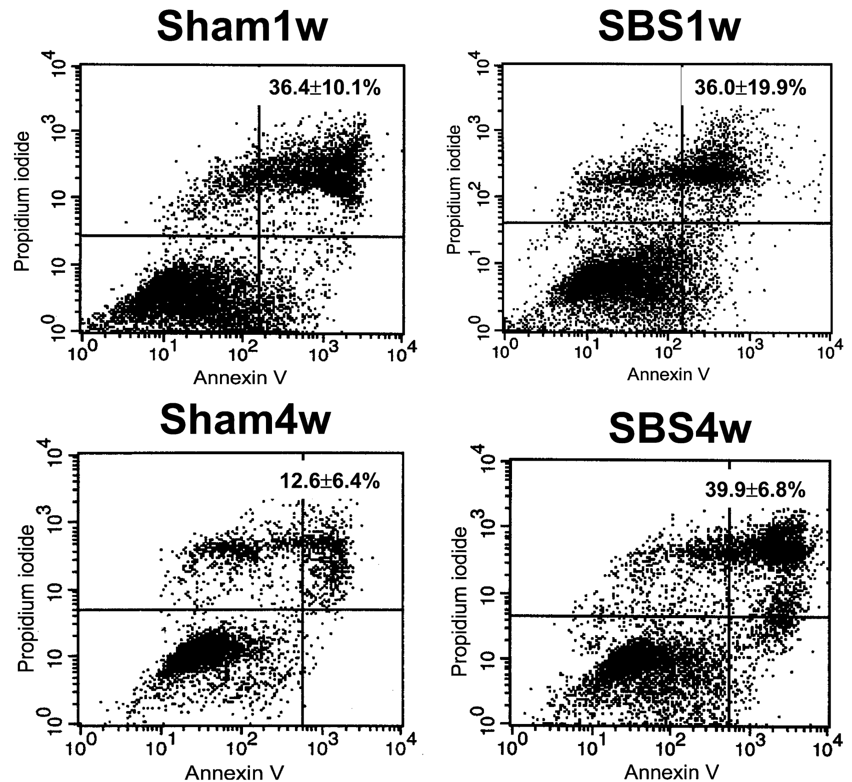

Fig. 2 Flow cytometric analysis of epithelial cell (*EC*) apoptosis. Apoptotic rate is detected by gating on EC with flow cytometry and detecting the percent of Annexin V staining as percentage of total EC. Cell necrosis is represented by propidium iodine positive cells, without Annexin V staining (*left upper quadrant*). Note the increase in EC apoptosis in mice 1 week postoperative and its decline 4 weeks postoperative in the sham group with persisting high rate in SBR4w mice. Necrosis showed no significant changes between groups

Table 3 IEL genes with greatest alterations in growth-modifying factors; shown are PCR results. [a]$P < 0.05$ Sham1w vs. SBR1w, [b] $P < 0.05$ Sham4w vs. SBR4w, [c] $P < 0.05$ Sham1w vs. Sham4w, [d] $P < 0.05$ SBR1w vs. SBR4w

Gene	Lipocalin 2	Angiotensin converting enzyme	Rap2 interacting protein	Amphiregulin	Leucine-rich-α2-glyco-protein
Function	Apoptosis↑	Apoptosis↑	Apoptosis↓ Proliferation↑	Proliferation↑	Apoptosis↑ Proliferation↓
Sham 1w mean	0.72 ± 0.65[c]	0.93 ± 0.10[a]	0.27 ± 0.17	0.31 ± 0.16	0.57 ± 0.28
SBR 1w mean	0.62 ± 0.13	1.04 ± 0.07	0.18 ± 0.13[d]	0.18 ± 0.07[d]	0.54 ± 0.11
Sham 4w mean	0.25 ± 0.22[b]	0.89 ± 0.06[c]	0.26 ± 0.08	0.25 ± 0.09	0.34 ± 0.24[b]
SBR 4w mean	0.45 ± 0.27	1.01 ± 0.24	0.21 ± 0.16	0.25 ± 0.11	0.47 ± 0.21

works to maintain equilibrium between cell proliferation and cell death. Apoptosis, a morphologically and biochemically distinct form of cell death, plays an essential biological role to maintain homeostasis and physiological integrity in most tissues. After massive small bowel resection (SBR) an adaptive response in the residual intestine with an increased EC-apoptosis and -proliferation rate has been described [4, 6, 7]. Our results confirmed these homeostatic changes.

However, the regulation of gastrointestinal growth remains unclear, but is known to be induced by many factors [8]. Intraepithelial lymphocytes (IEL) appear to play an important role in this intestinal homeostasis by secreting a variety of cytokines [1, 2, 3]. Among known IEL factors, KGF, IFN-γ, TNF-α or IL-12 are examples that may mediate proliferation, differentiation and apoptosis of EC [2, 9, 10, 11, 12]. Upon analysis of our microarray results we focused on expression of growth-related genes to determine further factors contributing to intestinal adaptation after SBR. Five IEL genes were significantly altered and, based on our results, there is reason to believe that they have a decisive impact on the adaptive response post-SBR.

Lipocalin 2 (LC2)

The LC family is a large group of small extracellular proteins that shows great functional diversity [13]. Only recently, induction of apoptosis has been revealed as one of the functions of LC2. In a published study on hematopoietic cells it was found that secreted LC2 induces and is required for apoptosis [14]. Also in our study, increase of LC2 expression was coincident with the increase in EC apoptosis. This finding supports this newly demonstrated LC2 capacity and suggests a so far unknown regulatory factor of intestinal growth regulation.

Rap2 interacting protein

Rap2 is a small GTP-binding protein that belongs to the Ras family, which is known to control various cellular functions [15]. Ras itself has been identified as a key regulator of both cell proliferation and differentiation [16], and is involved in decreasing apoptotic signals and regulating tissue maintenance [17]. Knowledge about Rap2 and Rap2 interacting protein is limited. However, it has been shown that Rap2 shares most of the effector proteins with Ras [18] and equates the function of this survival factor [19]. We showed a significant increase of Rap2 interacting protein in IEL of SBR4w mice, which indicates a possible function in the pathway of the regulation of intestinal homeostasis.

Amphiregulin (AR)

AR is one of the members of the epidermal growth factor (EGF) family and has been described as a potent stimulator of proliferation in a variety of cell types such as prostatic [20], mammary [21] and colonic epithelial cells [22]. AR has been shown to be present in a large number of both malignant and benign colon [22] and mammary tumors [23]. AR also seems to play an essential role in the mammary gland development, whereby AR deficiency leads to impaired mammary gland function [24]. Our results show an increase of AR expression in SBR4w mice, matching the observed increase in EC-proliferation and suggesting a possible role in intestinal adaptation.

Leucine-rich-α2-glycoprotein (LRGP)

Knowledge about the function of LRGP is scarce. However, in a recent study it has been shown that LRGP binds transforming growth factor beta (TGF-β) and may equally be involved in its functional pathway. TGF-β has been found to be a pro-apoptotic factor in the murine intestinal mucosa [25], as well as an inhibitor of cell proliferation [26]. LRGP expression was increased in SBR4w mice, at which time the EC-apoptotic rate was increased. However, our finding of increased proliferation in these mice is not consistent with the anticipated inhibition of cell proliferation by LRGP, assuming that the TGF-β pathway is followed. Because of the limited information about LRGP, it may not yet be possible to draw conclusions.

Angiotensin-converting enzyme (ACE)

ACE has been found to be a mediator in the induction of apoptosis of alveolar epithelial cells [27], as well as in non-epithelial cells like myocytes [28] and endothelial cells [29]. The mechanism by which ACE affects apoptosis is not yet clearly understood and seems to include different pathways. Several studies have described that with the use of ACE-inhibitors there is a change in the cytokine expression in lymphocytes [30, 31]. This implies that ACE may directly induce altered cytokine expression, potentially in the IEL, which thereafter may induce apoptosis in EC. ACE may also work via the renin-angiotensin system by effecting angiotensin II (ANG II), the product of the conversion of ANG I to II by ACE. ANGII may induce apoptosis, as seen in alveolar epithelial cells [27]. ACE also acts on the kallikrein-kinin system, where bradykinin, which is degraded by ACE, has been shown to have an impact on apoptosis in endothelial cells and cardiocytes [32, 33]. Recent studies focused on controlling ACE and its biological effects utilizing well-established ACE-inhibitors (ACE-I). The results are remarkable: administration of ACE-inhibitors clearly reduced the apoptotic rate in the abovementioned cell types [34, 35, 36]. In our study, the alterations of the ACE-mRNA expression preceded the observed apoptotic changes. However, it can be anticipated that ACE may have a regulatory function regarding apoptosis. These findings suggest that administration of an ACE-inhibitor to SBR mice would be beneficial. ACE-inhibitors may decrease the apoptotic rate of EC and promote the adaptive process after SBR, support compensation for the loss of mucosal surface area and basically prevent the short bowel syndrome.

These preliminary data show that the IEL undergo marked temporal changes after SBR. Clearly, more investigation is needed to further elucidate our findings. Nevertheless, our results evidently provide novel insight into potential IEL-dependent regulation of EC homeostasis after massive SBR.

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