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ORIGINAL ARTICLE

Accelerated cell turnover 48 h after intestinal ischemia is NOTCH independent

Y. Ben-Shahar[2,3] · Z. Abassi[3] · Y. Pollak[1] · A. Bitterman[3] · H. Kreizman-Shefer[3] · T. Koppelman[2] · A. E. Fuhrer[2] · L. Hayari[3] · I. Sukhotnik[1,2]

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Abstract

Aim of the study Notch signaling plays important roles in maintaining intestinal epithelial homeostasis. When Notch signaling is blocked, proliferation ceases and epithelial cells become secretory. The purpose of the present study was to evaluate the role of Notch signaling pathway following intestinal ischemia–reperfusion (IR) injury in a rat model.

Materials and methods Male Sprague–Dawley rats were randomly divided into four experimental groups: Sham-24 and Sham-48 rats underwent laparotomy and were killed 24 or 48 h later, respectively; IR-24 and IR-48 rats underwent occlusion of SMA and portal vein for 30 min followed by 24 or 48 h of reperfusion, respectively. Enterocyte proliferation and enterocyte apoptosis were determined at killing. Notch-related gene and protein expression were determined using Real Time PCR, Western blotting and immunohistochemistry 48 h followed IR.

Main results IR-48 rats demonstrated significantly increased rates of cell proliferation and increased cell apoptosis in both jejunum and ileum compared to Sham rats. IR-48 rats exhibited a significant decrease in Notch-1 protein expression (Western blot) that was coincided with a significant decrease in the number of Notch-1 positive cells (immunohistochemistry) in jejunum (35% decrease, $p < 0.05$) and ileum (twofold decrease, $p < 0.05$) as well as Hes-1 positive cells in jejunum (28% decrease, $p < 0.05$) and ileum (31% decrease, $p < 0.05$) compared to Sham-48 rats.

Conclusions Forty-eight hours following intestinal IR in rats, accelerated cell turnover was associated by inhibited Notch signaling pathway. Intestinal stem cells differentiation toward secretory progenitors rather than differentiation toward absorptive cells is important at this phase of intestinal recovery.

Keywords Intestine · Ischemia–reperfusion · Notch · Stem cells

Introduction

Intestinal ischemia–reperfusion (IR) injury is a combined process in which damage to the tissue is caused by hypoxia and by reactive oxygen radicals [1]. It is characterized by dysfunction of the gut barrier, which leads to increase in permeability of the epithelium [2] and infiltration of intestinal wall by inflammatory cells. These cells cause systemic inflammatory response by releasing pro-inflammatory cytokines and finally may result in multiple organ failure and death [3, 4]. Nowadays, programmed cell death known as apoptosis, which proceeds during reperfusion, has an essential role in cell death process following IR [5, 6].

Intestinal cell turnover is a regeneration process balanced by the proliferation and differentiation of enterocytes and by cell death due to apoptosis. This process is regulated through interactions between the epithelium and underlying mesenchymal stroma. Stem cells situated deeply in intestinal crypts proliferate, migrate and differentiate to become mature intestinal epithelium and form the intestinal villi [7]. These cells are regulated by four main signalling pathways, which include Wnt/b-catenin, hedgehog, bone morphogenetic protein (BMP) and Notch pathways [8].

✉ Y. Ben-Shahar
yoav_bs@hotmail.com; yohavbs@tlvmc.gov.il

[1] Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

[2] Department of Pediatric Surgery B, Dana-Dwek Children's Hospital, Tel Aviv Sourasky Medical Center, 6 Weizmann st, 6423906 Tel Aviv, Israel

[3] Department of Physiology, The Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel

Notch signalling pathway plays an important role in proliferation and differentiation of cells during maintenance of tissues homeostasis [9]. The Notch receptor–ligand complex in mammals is known as a family of four single trans-membrane Notch receptors (Notch1–4) and five single trans-membrane ligands Delta-like (DLL) 1, 3, 4, and Jagged (Jag) 1, 2 [9]. The activation of Notch receptor is initiated by binding to a ligand linked to a neighbouring cell. Juxtamembrane cleavage at S2 generates the membrane-anchored NEXT (Notch extracellular truncation) fragment, which serves as a substrate for the γ-secretase complex. γ-Secretase cleaves the Notch TMD progressively to release NICD (Notch intracellular domain) and Nβ peptides. When NICD enters the nucleus, its binding to CSL may trigger an allosteric change that facilitates displacement of transcriptional repressors. Evidences have suggested that HES-1, -5, -7, and HERP-1, -2, -3 are potential target genes of Notch [10]. Intestinal absorptive and secretory cells are non-dividing cells generated from intestinal stem cells through a series of transit-amplifying divisions. Enterocytes derive from an enterocyte precursor cell, while goblet cells, endocrine cells, and Paneth cells are thought to derive from a common secretory precursor cell [11, 12]. It is believed that Notch signalling acts to drive cell differentiation in the lining of the small intestine. When Notch signalling is blocked, proliferationstops and all cells become secretory [10, 12]. However, when Notch signalling is activated, proliferation of intestinal cells is somewhat extended, and there is no differentiation into secretory cells [13]. Transcription factor Hes-1 determines the specification of precursor cells into enterocyte fate, while the differentiation of secretory precursor cells into goblet and other secretory cells is regulated by Math1 and neurogenin 3 [14, 15].

The purpose of this study was to evaluate the role of Notch signalling pathway following intestinal ischemia–reperfusion (IR) injury in a rat model.

Materials and methods

Animals

The study was performed in accordance with the standards for handling and treating animals as defined in the Guide for the Care and Use of Laboratory Animals (Rappaport Faculty of Medicine, Technion, Haifa, Israel). Male Sprague–Dawley rats weighing 250–300 g were held in pairs in a 12-h day and night cycles at 21 °C for 3–5 days before the experiment. The animals were fed with standard chow and had free access to water. Animals were fasted for 24 h before the operation, but were allowed free access to water.

Experimental design

Rats were divided randomly into four experimental groups: Group I-Sham-24 rats underwent laparotomy and were killed 24 h after operation; Group II-IR-24 underwent laparotomy during which both SMA and PV were occluded for 20 min followed by 24 h of reperfusion; Group III-Sham-48 rats underwent laparotomy and were killed 48 h after operation, and Group IV-IR-48 rats underwent occlusion of both SMA and PV for 20 min followed by 48 h of reperfusion. The animals were killed 24 or 48 h after operation using intraperitoneal injection of pentobarbital (45 mg/kg) and open pneumothorax.

Surgical procedure

The rats were anaesthetized with an intraperitoneal injection of ketamine (90 mg/kg) and xylasine (15 mg/kg) after an overnight fast. A midline incision was made in the abdomen. In sham animals, the SMA and PV were identified and isolated but were not occluded. In IR animals, the SMA and PV were occluded using atraumatic microvascular clamps for 20 min. During the time of ischemia the abdominal wall incision was covered by wet gauze to prevent fluid and heat loss. Following 20 min of intestinal ischemia the incision was uncovered, the clamp was removed, and the ischemic gut was returned back into the abdomen. The abdomen was rinsed with a 3-ml intraperitoneal injection of warm 0.9% saline before closure. A running suture of 3/0Vicryl in two layers (Ethicon Corporation, USA) was used to close the abdominal cavity in all operations. The rats were only allowed free access to water 6 h following surgery. All animals were killed 24 (Groups I and II) or 48 (Groups III and IV) hours after operation. The small bowel was quickly removed, rinsed with cold isotonic saline and parted into two segments: proximal jejunum and terminal ileum. Each segment was weighed, cut longitudinally and the mucosa was scraped using a glass slide, collected and weighed. Histologic sections were prepared from the proximal jejunum and distal ileum by fixation in 4% buffered formalin for 24 h and processed into standard paraffin blocks.

Immunohistochemistry

Standard 5-bromodeoxyuridine (5-BrdU) labelling reagent (Zymed Lab, Inc, CA) was injected at a dose of 1 ml per 100 g body weight to the animals 90 min before killing for detection of enterocyte proliferation. Five-micrometer paraffin-embedded slices (5 μm) were deparaffinized with xylene, rehydrated with graded alcohol, and stained with a biotinylated monoclonal anti-BrdU antibody system using

BrdU Staining Kit (Zymed Lab, Inc, CA). A ratio of crypt cells staining positively for BrdU per 10 crypts defines the proliferation index. Apoptotic cells were identified by performingimmunohistochemistry for Caspase-3 (Caspase-3 cleaved concentrated polyclonal antibody; dilution 1:100; Biocare Medical, Walnut Greek, CA). A combination of streptovidin–biotin–peroxidase method and microwave antigen retrieval on formalin-fixed, paraffin-embedded tissues was used according to the manufacturers' protocols. The number of stained cells was counted in at least ten villi in areas without necrosis for each group. The number of apoptotic cells per ten villi defies the apoptotic index.

In order to detect Notch signalling pathway, immunohistochemistry for Notch-1 (Notch-1 polyclonal antibody; dilution 1:100, ab 52627, Abcam, Cambridge, UK) and Hes-1 (Hes-1 polyclonal antibody; dilution 1:100, ab 71559, Abcam, Cambridge, UK) was performed using a combination of the streptovidin–biotin–peroxidase method and microwave antigen retrieval on formalin-fixed, paraffin-embedded tissues according to the manufacturer's protocols.

Real-time PCR

Total RNA was isolated by Trizol (Invitrogen) reagent according to manufacturer's protocol. RNA was quantified using 260/280 nm spectrophotometry. Then, 500 ng of total RNA was converted by reverse transcriptase (qScriptcDNA Synthesis Kit Quantabio, USA) into complementary DNA (cDNA) which was then amplified by PCR-Thermal Cycler (2720 Thermal Cycler, ABI, Israel). Quantitative real-time PCR ABI-PRISM 7000 (applied Biosystems, Foster City, CA) determined gene expression of Notch-1, Jag1, Jag2, DKK1 and DLL1 mRNA on cDNA samples using PerfeCTa SYBR Green FastMix, Low ROX (Quantabio, USA) with the exception of template and primers.

Western blotting

Rat's jejunal and ileal tissue samples were homogenized in RIPA lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 2 mM EDTA, supplemented with a cocktail of protease (Roche Diagnostic) and phosphatase cocktail inhibitors (Sigma) for protein extraction.

The homogenate was centrifuged at 7500 rpm at 4 °C for 15 min and the supernatant was collected. Bradford reagent was used to determine protein concentrations according to manufacturer's instructions. Samples comprising same amounts of total protein (30 µg) were resolved by SDS–PAGE under reducing conditions. After electrophoresis, proteins were transferred to PVDF membrane and tested with anti-Notch-1 antibody (dilution 1:100, ab 52,627), and anti-Hes-1antibody (dilution 1:100, ab 71,559). Horseradish peroxidase-conjugated secondary antibody was purchased from Jackson Immuno Research Laboratories Inc. (West Grove, PA) and an enhanced chemiluminescent substrate from Biological Industries (Kibbutz Beth Ha-Emek, Israel).

Statistical analysis

The data are expressed as mean ± SEM. Kruskal–Wallis test was used for statistical analysis of parameters related to adaptation, enterocyte proliferation, and apoptosis, followed by post hoc test for multiple comparisons. Parameters were considered statistically significant if p values wereless than 0.05.

Results

Intestinal epithelial cells proliferation and apoptosis

We introduced in our previous study [16] that rats showed a significant decrease in proliferation of intestinal epithelial cells 24 h after intestinal IR in both jejunum and ileum compared to control animals. In the current study, we have demonstrated that 48 h after IR injury rats exhibited a slight but significant increase in proliferation rates of intestinal epithelial cells in jejunum (12% increase, $p < 0.05$) and ileum (13% increase, $p < 0.05$) compared to sham animals that were also significantly higher compared to IR-24 rats (Fig. 1). We have also demonstrated a significant increase in the number of apoptotic cells in the villi of jejunum (threefold increase, $p < 0.05$) and ileum (threefold increase, $p < 0.05$) in IR rats compared to sham animals (Fig. 1). However, the total number of apoptotic cells was lower in IR-48 group in comparison to IR-24 group.

Notch signalling related genes (Real-Time PCR)

Determination of Notch-related gene expressions by real-time PCR (Fig. 2) discovers an upward trend in expression of Notch-1 (13% increase), DLL4 (9% increase), Jag1 (20% increase) and a significant increase in Jag2 (60% increase, $p < 0.05$) mRNA levels in jejunum of IR-48 rats compared to sham animals.In ileum, a slight downward trend was demonstrated in Notch-1 (18% decrease), DLL4 (34% decrease), Jag2 (3% decrease) except a significant increase in expression of Jag1 (32% increase, $p < 0.05$) mRNA levels in IR-48 rats compared to sham animals.

Notch signalling related protein levels (Western blot)

In contrast to the upward trend of Notch signalling related genes expression, ischemic rats (Group D) demonstrated a significant decrease in Notch-1 protein expression in

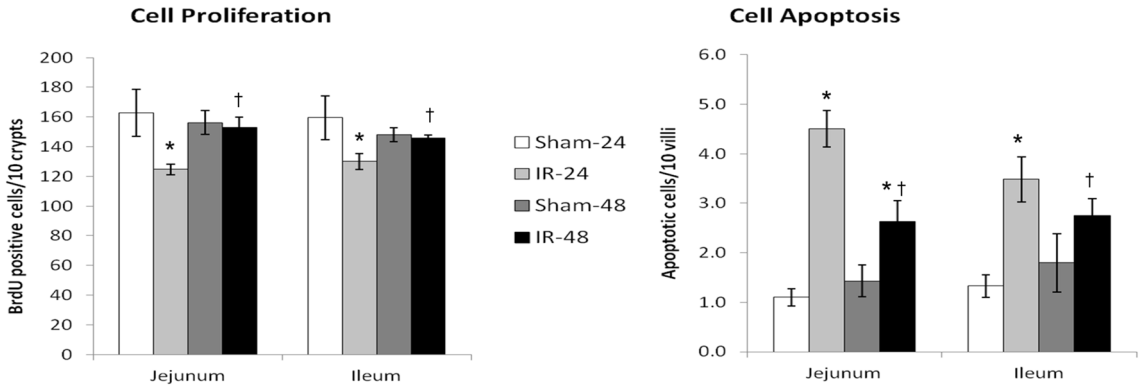

Fig. 1 Effect of intestinal ischemia–reperfusion on intestinal cell proliferation and apoptosis 24 and 48 h following laparotomy (Sham) or interstinal IR. Values are mean ± SEM. *IR* ischemia–reperfusion. *$p < 0.05$ IR rats vs sham rats. †$p < 0.05$ IR 48 vs IR 24

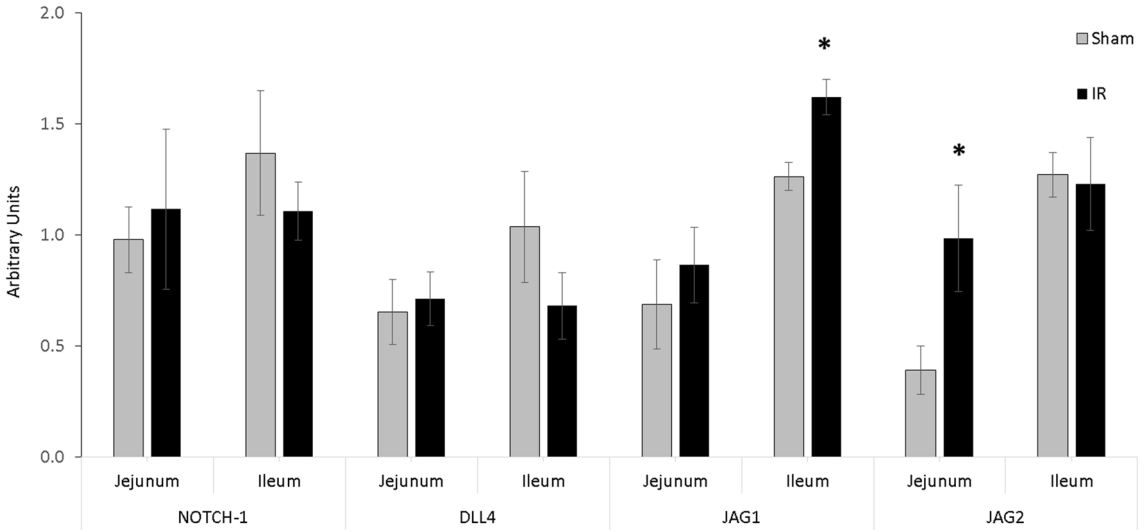

Fig. 2 Effect of intestinal ischemia–reperfusion on Notch signalling related genes. Gene Expression of Notch-1, DLL4, Jag1, and Jag2 mRNA was determined by quantitative real-time PCR. Values are mean ± SEM. *IR* ischemia–reperfusion. *$p < 0.05$ IR rats vs sham rats

both jejunum (six-fold decrease, $p < 0.05$) and ileum (37% decrease, $p < 0.05$) as well as trend toward decrease in Hes-1 protein in jejunum (24% decrease, $p = 0.07$) in IR rats compared to sham animals (Fig. 3). The significant down-regulation of Notch-1 protein expression was accompanied with a trend toward increase in Adam 17 protein levels in both jejunum and ileum; however, this trend was not statistically significant.

Immunohistochemistry

A significant decrease in the number of Notch-1 positive cells was demonstrated in jejunal (32% decrease, $p < 0.05$) and ileal (37% decrease, $p < 0.05$) villi of IR-48 rats compared to Sham-48 animals. In addition, IR-48 rats have shown a significant decrease (vs Sham-48 animals) in the number of Hes-1 positive cells in the villi of jejunum (29% decrease, $p < 0.05$) as well as a trend toward decrease in the number of Hes-1 positive cell ileum (28% decrease); however, this trend was not statistically significant. Regarding the number of Notch-1 positive cells in the crypts, a significant decrease in the number of positive cells was observed in the ileal crypts (44% decrease, $p < 0.05$) of IR-48 rats compared to sham animals (Figs. 4, 5).

Discussion

Intestinal epithelial cells regeneration following ischemia–reperfusion injury which is known also as intestinal cell turnover relies in particular on activity of intestinal stem cells (ISCs). Multiple signaling pathways

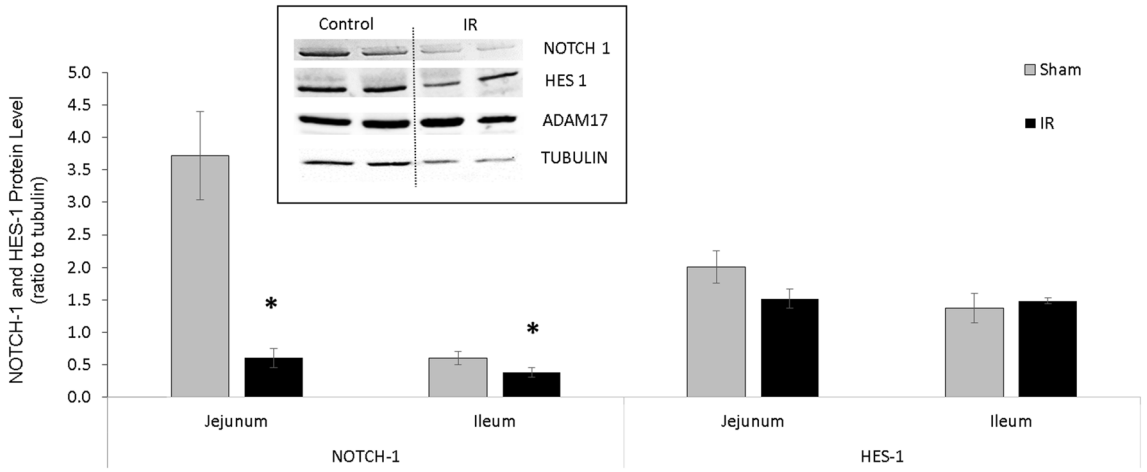

Fig. 3 Effect of intestinal ischemia–reperfusion on Notch-1 and Hes-1 protein expression. Western blot was used to determine protein levels. Values are mean ± SEM. *IR* ischemia–reperfusion. *$p < 0.05$ IR rats vs sham rats

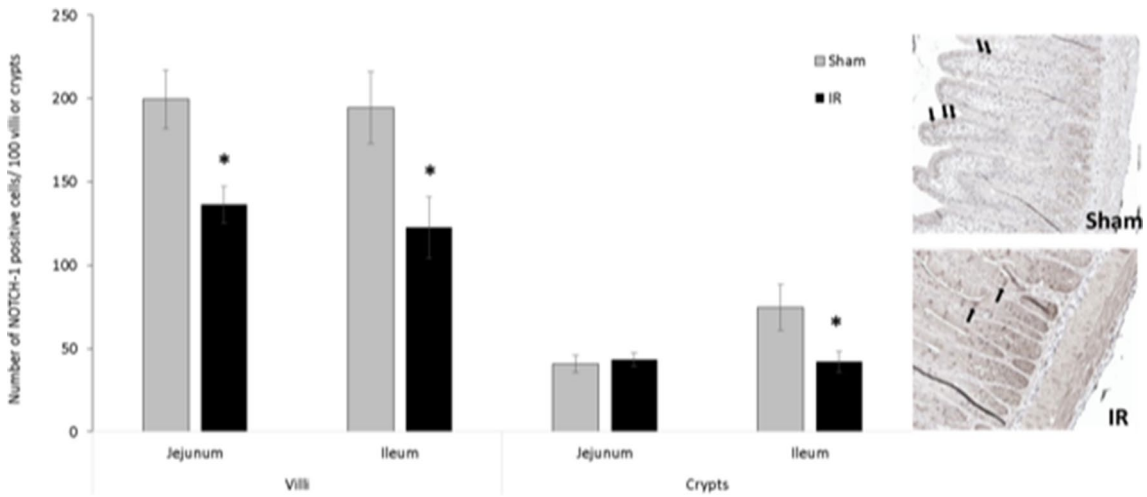

Fig. 4 Immunohistochemistry for Notch-1 was performed for identification of positive cells using a combination of the streptovidin-biotin–peroxidase method and microwave antigen retrieval on formalin-fixed, paraffin-embedded tissues according to the manufacturer's protocols. Values are mean ± SEM. *IR* ischemia–reperfusion. *$p < 0.05$ IR rats vs sham rats

regulate an unceasing process of proliferation, differentiation, and apoptosis of intestinal epithelia. Intestinal stem cells are capable to differentiate into special cells occupying intestinal villi like enterocytes, endocrine cells, and goblet cells. Under conditions of stress like IR injury, this regeneration process is augmented as stem cells divide and give rise to two progenitor cells that substitute injured intestinal cells. Several studies investigated the regulation of intestinal stem cell have suggested that Wnt/β-catenin, BMP (bone morphogenic protein), Notch, and PI3K/PTEN (P-phosphatase and tensin homologue) signaling pathways are involved in the regulation of intestinal stem cell proliferation and differentiation [8, 17, 18].

Notch signaling pathway has a main role as a regulator of tissue homeostasis and stem cell activity including in the gastrointestinal tract [18]. Notch receptors and ligands as Notch1, Jagged1, and Jagged2 are expressed in various proliferative compartments of the GI tract and function to sustain populations of gut cells in an undifferentiated state [19]. Previous studies indicated that Notch signaling promotes differentiation of progenitor cells into the absorptive ones rather than to secretory cells [20, 21]. It was demonstrated previously that using conditional gene targeting of RBP-J or pharmacological γ-secretase inhibitors blocked the release of NICD and thereby inhibited the activity of Notch signaling in the intestinal epithelium. This inhibition resulted in

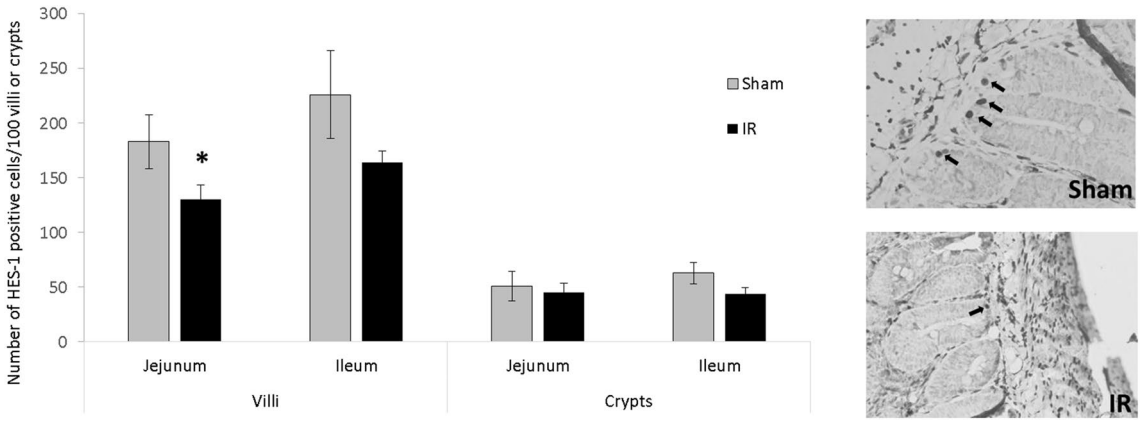

Fig. 5 Immunohistochemistry for Hes-1 was performed for identification of positive cells using a combination of the streptovidin-biotin–peroxidase method and microwave antigen retrieval on formalin-fixed, paraffin-embedded tissues according to the manufacturer's protocols. Values are mean ± SEM. *IR* ischemia–reperfusion. $^{*}p < 0.05$ IR rats vs sham rats

the loss of the proliferative cells and conversion of crypt progenitors into secretory cells [22]. Several studies have established the role of Notch signaling cascade as a key regulator of proliferation and differentiation of intestinal cells. Notch signaling promotes differentiation to the absorptive cell lineage rather than to the secretory cell lineage [23, 24]. However, the role of in stimulation of cell proliferation and differentiation after intestinal ischemia reperfusion injury has not been well established.

The purpose of this study was to evaluate the role of Notch signaling during intestinal recovery following intestinal ischemia–reperfusion injury in a rat model. We have demonstrated previously [16, 25] that IR rats demonstrated a significant increase in intestinal injury score 24 h following intestinal IR injury. This significant intestinal damage was accompanied by an intestinal mucosal hypoplasia. This was evident from decrease in bowel and mucosal weight as well as decrease in villus height and crypt depth compared to control animals. These findings suggested intestinal damage rather than intestinal recovery [25]. 48 h after intestinal IR, ischemic rats had less intestinal damage, but significantly increased mucosal weight and villus height compared to IR-24 animals, suggesting intestinal recovery. The current study is a continuation of our previous experiment. We have shown that cell proliferation increased significantly in jejunum and ileum of IR-48 rats compared to sham group that may suggest increased stem cell activity. This increase in intestinal epithelial cells proliferation was accompanied by decrease in the rate of cell apoptosis suggesting accelerated intestinal epithelial cell turnover.

Next, we determined the role of Notch signaling in regulating the activity of intestinal stem cells after intestinal IR by exploring Notch signaling related gene expression in real-time PCR. We have demonstrated that 48 h following IR event, accelerated intestinal epithelial cell turnover was accompanied by an upward trend in Notch-1, DLL4, Jag-1and a significant increase in Jag2mRNA levels in jejunum compared to sham animals. In ileum, we have demonstrated a slight downward trend in Notch-1, DLL4, Jag2 except a significant increase in the expression of Jag1 mRNA levels in IR-48 rats compared to sham animals.

The upward trend of Notch-related genes expression was in contrast to a significant down-regulation of Notch-1 protein levels in jejunum and ileum as well as in a significant decrease in Hes-1 protein level in jejunum in IR-48 rats compared to sham animals. This decrease in Notch-1 and Hes-1 protein levels was accompanied by a trend toward increase in Adam 17 protein levels in both jejunum and ileum; however, this trend did not achieve statistical significance.

Immunostaining for Notch-1 and Hes-1 positive cells in intestinal villi 48 h following IR injury exhibited a significant decrease in the number of Notch-1 positive cells in jejunum and ileum compared to sham animals. Also the number of Hes-1 positive cells decreased significantly in the villi of jejunum and in ileum in ischemic rats compared to control animals, but this change was not statistically significant. The number of Notch-1 and Hes-1 positive cells in jejunal crypts was not changed between ischemic and sham groups, however in ileal crypts there was a significant decrease in Notch-1 and Hes-1 positive cells in the IR-48 group compared to sham animals.

Notch family related genes consist of four Notch genes and five genes encoding ligands, three Delta-like (DLL) and two Jagged. Genetic studies confirmed that ablation of DLL4 in the intestinal epithelium does not perturb proliferation or goblet cell differentiation in the crypt. The studies showed that DLL4-mediated Notch signaling is indeed occurring in the intestine and that the absence of a measurable phenotype in DLL4 mutant mice is due to redundant DLL4-mediated Notch signaling [18]. There are

six known target genes of Notch signaling: HES-1, -5, -7, and HERP 1–3. The HES family is a basic helix-loop-helix type transcriptional repressor and acts as Notch effectors by negatively regulating expression of downstream target genes such as tissue-specific transcription factors [26]. Thus, HES/E(spl) directly acts as a primary Notch effector by affecting cell fate decisions. 48 h following intestinal ischemia–reperfusion resulted in an up-regulation trend of Notch-related genes in jejunum and down-regulation in ileum. Regarding Notch-related protein levels, there was a significant decrease in Notch-1 protein level in both jejunum and ileum and a trend toward decrease in Hes-1 protein level in jejunum in IR-48 rats compared to control animals. A concomitant up-regulation of Adam protein has been demonstrated in ischemic rats compared to sham animals. Notch binding to ligand elicits several steps of cleavage. The first one at the S2 site is mediated by the proteases ADAM10 or by TACE (TNF-a-converting enzyme). This catalyzes the processing of Notch in the intramembranous S2 and S3 sites by the γ-secretase complex.

Taking all results together raises our hypothesis that 48 h following intestinal ischemia–reperfusion injury Notch signaling cascade is still inhibited. This is evident from decrease in Notch-1 and Hes-1 protein expression of and concomitant decrease in the number of Notch-1 and Hes-1positive cells (by immunohistochemistry). However, besides enhanced intestinal cell turnover characterized by an increase in proliferation of intestinal epithelial cells and a decrease in the rate of apoptotic cells, there are signs of beginning activation of Notch signaling cascade. This activation is characterized by increase in Notch-related gene expression particularly in the jejunum and by increase in Notch-related proteins, like Notch effector protein Hes-1 in the ileum and the protease Adam 17 which responsible on cleavage process of Notch transmembrane receptor in both ileum and jejunum.

In conclusion, accelerated cell turnover is associated by inhibited Notch signaling pathway forty-eight hours following intestinal ischemia–reperfusion injury in rats. Still, preliminary signs of Notch signaling cascade activation emerge. Inhibition of Notch signaling cascade at this phase leads to intestinal stem cells differentiation toward secretory progenitors rather than differentiation toward absorptive cells which is important of intestinal recovery.

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