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www.wjgnet.com
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• *BASIC RESEARCH* •

Acid fibroblast growth factor reduces rat intestinal mucosal damage caused by ischemia-reperfusion insult

Wei Chen, Xiao-Bing Fu, Shi-Li Ge, Tong-Zhu Sun, Wen-Juan Li, Zhi-Yong Sheng

Wei Chen, Xiao-Bing Fu, Tong-Zhu Sun, Wen-Juan Li, Zhi-Yong Sheng, Wound Healing and Cell Biology Laboratory, Burns Institute, 304th Clinical Department, General Hospital of PLA, Beijing 100037, China
Shi-Li Ge, Institute of Radiation Medicine, Academy of Military Medicine Sciences, Beijing 100850, China
Supported by the National Natural Science Foundation of China, No. 30400172 and 30230370 and "973" programm NO.2005CB52203
Correspondence to: Professor Xiao-Bing Fu, Wound Healing and Cell Biology Laboratory, Burns Institute, 304th Clinical Department, General Hospital of PLA, 51 Fu Cheng Road, Beijing 100037, China. fuxb@cgw.net.cn
Telephone: +86-10-66867396 Fax: +86-10-88416390
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Abstract

AIM: To detect the effects of acid fibroblast growth factor (aFGF) on apoptosis and proliferation of intestinal epithelial cells in differentiation or proliferation status to explore the protective mechanisms of aFGF.

METHODS: Wistar rats were randomly divided into sham-operated control group (C, $n = 6$), intestinal ischemia group (I, $n = 6$), aFGF treatment group (A, $n = 48$) and intestinal ischemia-reperfusion group (R, $n = 48$). Apoptosis of intestinal mucosal cells was determined with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) technique. Proliferating cell nuclear antigen (PCNA) protein expression and distribution were detected with immunohistochemical method. Plasma levels of D-lactate were determined with modified Brandts method.

RESULTS: In A group, administration of exogenous aFGF could improve intestinal histological structure and decrease plasma D-lactate levels at 2-12 h after the reperfusion compared with R group. The apoptotic rates and PCNA protein expressions were not increased until 2 h after reperfusion and were maximal at 12 h. After reperfusion for 2-12 h, the apoptotic rates were gradually augmented along the length of jejunal crypt-villus units. Administration of aFGF could significantly reduce the apoptotic response at 2-12 h after reperfusion ($P<0.05$). Apoptosis rates in villus and crypt epithelial cells in A group at 12 h after reperfusion were (62.5±5.5)% and (73.2±18.6)% of those in R group, respectively. Treatment of aFGF could apparently induce protein expression of PCNA in intestinal mucosal cells of A group compared with R group during 2-12 h after reperfusion ($P<0.05$). There were approximately 1.3- and 1.5-times increments of PCNA expression levels in villus and crypt cells in A group at 12 h after reperfusion compared with R group, respectively.

CONCLUSION: Intestinal I/R insult could lead to histological structure change and apoptotic rate increment. The protective effects of aFGF against ischemia/reperfusion in rat intestinal mucosa might be partially due to its ability to inhibit ischemia/reperfusion-induced apoptosis and to promote cell proliferation of crypt cells and villus epithelial cells.

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Key words: Acid fibroblast growth; Ischemia; Reperfusion; Intestine; Crypt; Villus

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INTRODUCTION

An ischemic insult decreases oxygen and nutrient delivery and causes post mitotic cell lineages in the brain, myocardium, muscle and adrenal cortex to undergo apoptosis. Paradoxically, the introduction of oxygen during reperfusion of ischemic tissues exacerbates the damage of these tissues[1-5]. These tissues do not allow evaluation of the effects of proliferative status or state of differentiation on the apoptotic response to this inductive stimulus. The intestinal epithelium provides an excellent system for such an analysis. Renewal of intestinal epithelium takes place continuously in anatomically distinct crypt-villus units. Proliferation is restricted to mucosal invaginations known as crypts. All epithelial cells in each crypt are derived from an uncertain number of multipotent stem cells located at or near the base of the crypt[6-8]. These crypt stem cells divided to produce daughter stem cells as well as more rapidly replicating transit cells, which in turn undergo 4-6 rapid cell divisions in the proliferative zone located in the lower half of each crypt[9,10]. The small intestine is highly sensitive to ischemia-reperfusion (I/R). It has been demonstrated that occlusion of the superior mesenteric artery (SMA) followed by reperfusion can cause apoptosis in the intestinal epithelium[11].

Acid fibroblast growth factor (aFGF) is a mitogen *in vitro* for most of the ectodermal- and mesodermal-derived cell lines. In addition, this factor shows a wide range of endocrine-like activities[12,13]. As a multiple function growth factor, aFGF is involved in embryo development and tissue repair[14-16]. However, the protective mechanisms of aFGF on intestinal I/R injury remain unknown. Given the anatomically well-defined stratification of proliferation and differentiation programs along its crypt-villus axis, the self-renewing intestinal epithelium provides an opportunity to consider, whether aFGF protects intestinal mucosa from I/R injury through inducing proliferation of cells located in crypt or inhibiting apoptosis of epithelial cells distributed in villus or adopting both these two procedures.

In the present article, we have clarified the mechanism of aFGF protection following I/R injury in the small intestinal mucosa of rats by evaluating changes in apoptosis of epithelial cells along gut crypt-villus axis. We evaluated the expression of proliferating cell nuclear antigen (PCNA). The expressive level of this protein is frequently used to evaluate the regenerative ability of epithelial tissue[17]. We also estimated the integrity of small intestinal mucosa by measuring the levels of plasma D-lactate. This study will lead to a better understanding of the repair mechanisms of small intestinal mucosa induced by aFGF treatment after I/R injury.

MATERIALS AND METHODS

Animal model and experimental design

Healthy male Wistar rats weighing 220 ± 20 g (Animal Centre, Academy of Military Medical Science, Beijing) were used in this study. Animals were housed in wire-bottomed cages placed in a room illuminated from 08:00 am to 08:00 pm (12:12 h light-dark cycle) and maintained at 21±1 °C. Rats were allowed to access water and chow *ad libitum*. The animals were anesthetized by using 3% sodium pentobarbital (40 mg/kg), and a laparotomy was performed. The SMA was identified and freed by blunt dissection. A micro-bulldog clamp was placed at the root of SMA to cause complete cessation of blood flow for 45 min, and thereafter the clamp was loosened to form reperfusion injury. The animals were randomly divided into sham-operated control group (C, $n = 6$), intestinal ischemia group (I, $n = 6$), aFGF treatment group (A) and intestinal ischemia-reperfusion group (R). According to the different periods after reperfusion, groups R and A were further divided into 0.25, 0.5, 1, 2, 6, 12, 24 and 48 h subgroups, respectively ($n = 6$, each subgroup). In group I, the animals were killed after 45 min of SMA occlusion, while in groups R and A, the rats sustained 45 min of SMA occlusion and were treated with 0.15 mL normal saline and 0.15 mL saline plus 20 μg/kg aFGF (R&D Systems, Inc.) injected from tail vein, respectively, then sustained 0.25, 0.5, 1, 2, 6, 12, 24 and 48 h of reperfusion, respectively. In C group, SMA was separated, but without occlusion, and the samples were taken after exposure of SMA for 45 min. In groups R and A, rats were killed at different time points after reperfusion. Blood samples and intestinal tissue biopsies were taken. Blood samples were centrifuged and serum was frozen to measure plasma levels of D-lactate. Tissue biopsies were fixed with 10% neutral buffered formalin for detection of intestinal epithelial apoptosis, and protein expression of PCNA.

In situ detection of cell death

The apoptotic cells in intestinal tissues were detected with the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) method. Specimens were dewaxed and immersed in phosphate-buffered saline containing 0.3% hydrogen peroxide for 10 min at room temperature and then incubated with 20 μg/mL proteinase K for 15 min. Seventy-five microliters of the equilibration buffer was applied directly onto the specimens for 10 min at room temperature, followed by 55 μL of TdT enzyme and incubation, which were then incubated at 37 ℃ for 1 h. The reaction was terminated by transferring the slides to prewarmed stop/wash buffer for 30 min at 37 ℃. The specimens were covered with a few drops of rabbit serum and incubated for 20 min at room temperature and then covered with 55 μL of anti-digoxigenin peroxidase and incubated for 30 min at room temperature. Specimens were then soaked in Tris buffer containing 0.02% diaminobenzidine and 0.02% hydrogen peroxide for 1 min to achieve color development. Finally, the specimens were counterstained by immersion in hematoxylin. The cells with clear nuclear labeling were defined as TUNEL-positive cells. The results of positive cells and their distribution were observed under 400 times microscope. Sixty intestinal villi and crypts per time point were required for counting, and then the apoptotic ratios were calculated and analyzed, respectively.

Immunohistochemistry

Immunostaining for PCNA was performed in paraffin sections with a high-temperature antigen-unmasking method in citrate buffer and ABC peroxidase, using monoclonal mouse antibody (Zymed Corp., ZM-0213) against antigen (1 ∶ 100 in PBS). Tissues were fixed overnight in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections of 5 μm thickness were deparaffinized and rehydrated using graded alcohol concentrations. Antigen retrieval was performed by incubation in 100 mmol/L sodium citrate, pH 6.0, at 90 ℃ for 20 min. Then, sections were blocked with 5% normal swine serum in PBS for 30 min at 25 ℃, followed by incubation with primary antibodies at a concentration of 5 μg/mL overnight at 4 ℃. Control slides were incubated with PBS without primary antibodies. Tissues sections were then incubated for 60 min with biotinylated secondary antibody. After washing in PBS, the sections were exposed to acidin-biotin complex for 60 min. The sections were reacted with 0.05% (wt/vol) DAB in 50mol/L Tris-HCl (pH 7.4) with 0.1% (vol/vol) hydrogen peroxide for 5 min and counterstained with hematoxylin. The results of positive staining cells and their distribution were observed under 400 times microscope. Sixty intestinal villi and crypts per time point were required for counting,

and then the ratio of positive cells were calculated and analyzed, respectively.

Measurement of plasma D-lactate

The levels of plasma D-lactate were measured with modified Brandts method[18,19]. Briefly, heparinized blood was centrifuged at 3 200 r/min for 10 min and 2 mL of the plasma was deproteinized with 0.2 mL perchloric acid (1/10 vol), mixed and kept in an ice bath for 10 min. The denatured protein solution was centrifuged at 3 200 r/min for 10 min and the supernatant was removed. To 1.4 mL of supernatant, 0.12 mL KON was added and they were mixed for 20 s. Precipitant $KCLO_4$ was removed by centrifugation at 3 200 r/min for 10 min. The supernatant and neutralized-protein-free plasma were used to measure the absorbency at 304 nm. Plasma D-lactate concentrations were expressed as μg/mL.

Statistical analysis

All values were expressed as mean±SD. Differences in mean values were compared using SPSS 11.0 by one-way ANOVA and Student-Newman-Keul (SNK) test. $P<0.05$ as considered as statistically significant.

RESULTS

Change of cellular apoptotic rates

After the SMA was clamped near its origin from the aorta, the damage to the small intestine in the 45-min ischemia group was small. At 2 h after reperfusion, the partial loss of the mucosa could be observed. During 6-12 h after reperfusion, the damage of intestinal epithelial cells, hemorrhage and necrosis could be found, the crypt-villus structure was seriously spoiled. In the period of 24-48 h after reperfusion, the mucosal integrity was partially restored. The protective function of aFGF on intestinal mucosa was the most effective during 2-12 h after reperfusion. The structures of crypt and villus were both guarded, with less damage of the intestinal mucosa (Figure 1). Apoptosis was measured and quantified by TUNEL assay in serial sections prepared from the middle quarter of the small intestine (jejunum). The time animals were killed after a 45 min SMA occlusion followed by reperfusion was varied to further define the apoptotic response. Statistically significant increases in TUNEL-positive cells were not detectable until 2 h after reperfusion and were maximal at 12 h. The augmentation of the cellular apoptotic rates was evident along the length of jejunal crypt-villus units after reperfusion for 2-12 h; i.e., the average increase was 2.3 times for villi [84.0±6.2 (12 h after reperfusion) *vs* 37.3±6.6 (C group $P<0.05$)] and 1.9 times for crypts [28.2±3.9 (12 h after reperfusion) *vs* 14.4±3.8 (C group $P<0.05$; Table 1)]. Highest levels of apoptosis were noted in the upper quarter of the villus. In no matter villus and crypt, the apoptotic rates were restored to the levels of C group after reperfusion for 24 and 48 h. Administration of aFGF could significantly reduce the apoptotic response observed 2-12 h after reperfusion ($P<0.05$). Apoptosis rates in villus and crypt epithelial cells of A group at 12 h after reperfusion were (62.5±5.5)% and (73.2±18.6)% of those in their littermates of R group, respectively (Table 1, Figure 2).

Table 1 Effect of aFGF on the apoptotic rates in intestinal villi and crypts after ischemia-reperfusion insult ($n=6$, mean±SD, %)

Groups	Villus		Crypt	
	R group	A group	R group	A group
C group	37.3±8.6	37.3±8.6	14.4±3.8	14.4±3.8
I group	40.0±6.9	40.0±6.9	13.7±4.1	13.7±4.1
0.25 h after reperfusion	45.3±5.7	35.2±6.7[c]	15.0±3.2	11.8±2.6
0.5 h after reperfusion	46.3±8.3	37.8±7.4	16.2±4.7	13.0±3.5
1 h after reperfusion	49.5±5.3	45.3±7.4	19.3±5.6	16.2±3.3
2 h after reperfusion	70.2±6.5[a]	50.5±5.3[ac]	23.5±6.2[a]	17.8±4.1[c]
6 h after reperfusion	76.8±4.7[a]	49.2±5.0[a,c]	27.2±4.8[a]	19.3±5.0[a,c]
12 h after reperfusion	84.0±6.2[a]	52.5±4.6[a,c]	28.0±3.9[a]	20.5±5.2[a,c]
24 h after reperfusion	41.8±5.4	35.2±5.2	14.2±4.3	9.3±3.6
48 h after reperfusion	33.7±4.8	34.3±6.2	13.3±2.9	11.0±4.3

[a]$P<0.05$ *vs* C group; [c]$P<0.05$ *vs* R group at matched time points.

Expression characteristics of PCNA protein

Quantitative immunohistochemical results for PCNA protein were evaluated in Table 2 and Figure 3. PCNA was weakly expressed in the sham-operated intestinal tissues and ischemic tissues, with positive particles mostly

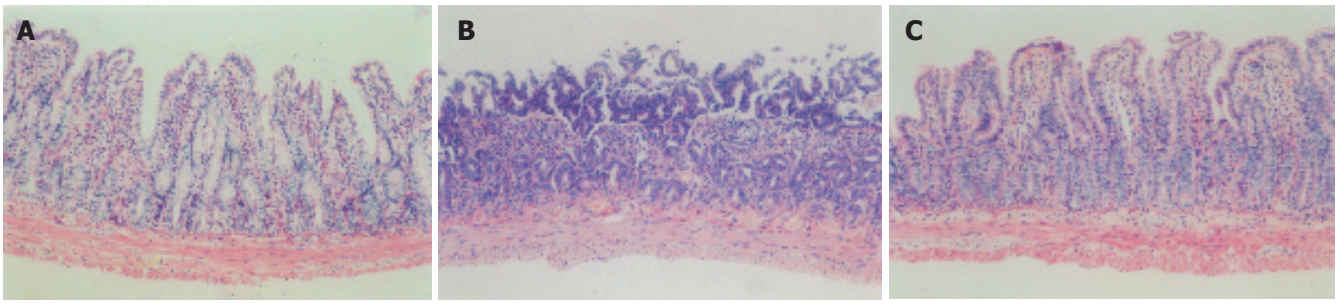

Figure 1 The histological structure of intestine in sham-operated control group **(A)**, 6 h after ischemia-reperfusion in R group **(B)** and 6 h after ischemia-reperfusion in A group **(C)** (100×). Anatomic structures of villi and crypts of intestine in control group were intact. In R group, at 6 h after reperfusion, the crypt-villus structure was seriously spoiled, accompanied by inflammatory cells infiltrating into the intestinal wall. In A group, the structures of crypt and villi were both protected, with less damage of the intestinal mucosa.

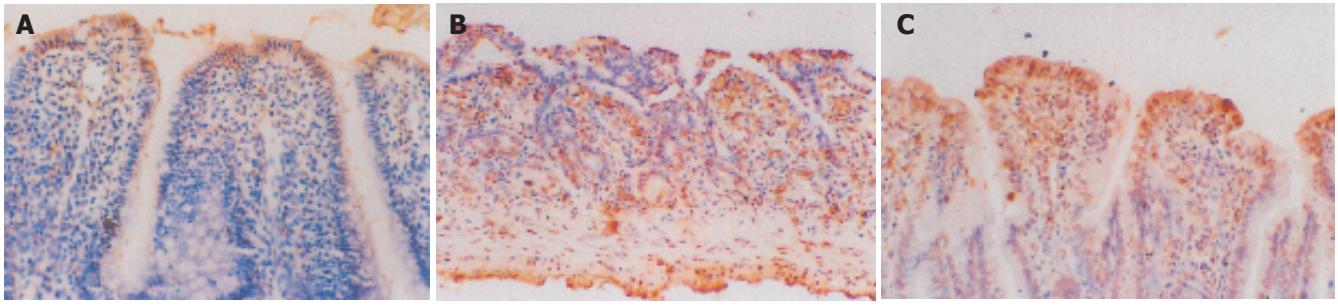

Figure 2 The cellular apoptotic rates in sham-operated control group **(A)**, 6 h after ischemia-reperfusion in R group **(B)** and 6 h after ischemia-reperfusion in A group **(C)** (200×). Cellular apoptotic rates were less in sham-operated control group and were significantly increased in villi and crypts at 6 h after reperfusion. The augmentation of the cellular apoptotic rates was evident along the length of jejunal crypt-villus units. The cellular apoptotic rates both decreased remarkably at 6 h after reperfusion in villi and crypts of A group *vs* those of R group.

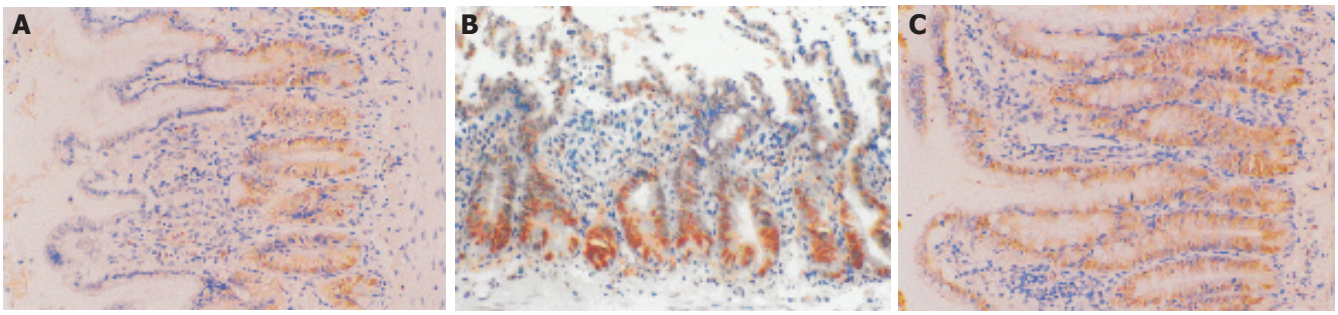

Figure 3 The expression of PCNA protein in sham-operated control group **(A)**, 6 h after ischemia-reperfusion in R group **(B)** and 6 h after ischemia-reperfusion in A group **(C)** (200×). PCNA was weakly expressed in sham-operated control group, with positive particles mostly distributing in the nuclei of the lower third of crypt cells. Protein contents of PCNA were significantly increased in villi and crypts at 6 h after reperfusion compared with control group. The contents of PCNA increased remarkably in villi and crypts at 6 h after reperfusion in A group *vs* R group.

Table 2 Effect of aFGF on protein expression of PCNA in intestinal villi and crypts after ischemia-reperfusion (n=6, mean±SD, %)

Groups	Villus		Crypt	
	R group	A group	R group	A group
C group	8.5±5.0	8.5±5.0	31.5±5.2	31.5±5.2
I group	8.8±5.3	8.8±5.3	33.3±4.1	33.3±4.1
0.25 h after reperfusion	10.5±5.1	10.7±4.9	39.7±5.4	36.0±5.6
0.5 h after reperfusion	11.5±3.8	14.7±4.3	41.0±4.7[a]	43.8±5.2[a]
1 h after reperfusion	12.3±6.2	11.3±5.4	44.3±4.6[a]	51.5±6.0[a]
2 h after reperfusion	16.7±4.5[a]	23.3±5.1[ac]	48.2±5.3[a]	67.8±5.3[ac]
6 h after reperfusion	18.2±3.5[a]	25.0±6.4[ac]	47.3±5.5[a]	76.0±3.8[ac]
12 h after reperfusion	19.0±5.4[a]	25.3±4.5[ac]	52.1±5.1[a]	79.8±5.2[ac]
24 h after reperfusion	8.7±4.7	11.2±2.3	43.7±5.4	44.8±6.2
48 h after reperfusion	6.7±2.9	9.2±4.0	38.5±6.1	42.7±4.9

[a]P<0.05 *vs* C group; [c]P<0.05 *vs* R group at matched time points.

distributing in the nuclei of the lower third of crypt cells. However, the positive cellular rate elevated with the increment of duration after reperfusion injury. In the period after reperfusion, marked PCNA immunoreactivities were observed in the nuclei of crypt and villus cell. At 12 h after reperfusion, the positive cellular rates were maximal, the average increase was 2.2 times for villi [19.0±5.4 *vs* 8.5±5.0 (P<0.05)] and 1.7 times for crypts [52.1±5.1 *vs* 31.5±5.2 (P<0.05; Table 2)]. The positive cellular rates of PCNA were restored to the levels of sham-operated tissues at 24 and 48 h after reperfusion. During 2-12 h after reperfusion, treatment of aFGF could apparently induce protein expression of PCNA in intestinal mucosal epithelial cells from villi and crypts in comparison with R group at different matched times (P<0.05) (Table 2). There are approximately 1.3- and 1.5-time increments of PCNA expressive levels in both villus and crypts in rats of A group after reperfusion for 12 h compared with their littermates of R group, respectively. After reperfusion for 24 and 48 h, the positive signals of this protein had no substantial change in A group in comparison with R group (P>0.05).

The changes of plasma levels of D-lactate

As shown in Table 3, 45 min of ischemia followed by reperfusion in intestinal mucosa could result in plasma D-lactate level elevation. Plasma D-lactate level was significantly elevated from 30 min after reperfusion in R group, and reach its peak at 12 h, which was 1.5-folds of that in sham-operated group ((0.299±0.025) μg/mL *vs* (0.201±0.008) μg/mL, P<0.05), and then returned to the level of sham-operated group (P>0.05). Administration of aFGF produced decreases in the plasmas D-lactate level compared with R group during 0.25-12 h after reperfusion, except for 1 h after reperfusion, leading to statistically significant reduction of D-lactate contents (P<0.05). No significant decreases were observed at 24 and 48 h after reperfusion in A group compared with R group

Table 3 Effect of aFGF on plasma D-lactate levels after intestinal ischemia-reperfusion (n=6, mean±SD, μg/mL)

Groups	R group	A group
C group	0.201±0.008	0.201±0.008
I group	0.212±0.011	0.212±0.011
0.25 h after reperfusion	0.247±0.011	0.225±0.016[c]
0.5 h after reperfusion	0.281±0.036[a]	0.222±0.020[c]
1 h after reperfusion	0.285±0.068[a]	0.233±0.022
2 h after reperfusion	0.283±0.031[a]	0.232±0.018[ac]
6 h after reperfusion	0.293±0.033[a]	0.252±0.014[ac]
12 h after reperfusion	0.299±0.025[a]	0.250±0.018[ac]
24 h after reperfusion	0.228±0.012	0.223±0.015
48 h after reperfusion	0.231±0.004	0.224±0.009

[a]P<0.05 *vs* C group; [c]P<0.05 *vs* R group at matched time points.

(P>0.05, Table 3).

DISCUSSION

The major clinical disorders involving gastrointestinal circulation are hemorrhage and ischemia. It is well recognized that the small intestine is very sensitive to the deleterious effects of I/R and it has been clearly demonstrated that I/R causes mucosal injury within the small intestine. Several recent studies have proposed that ischemia-reperfusion can induce an apoptotic response in the adult rat intestinal epithelium, although they did not quantitate this response along the crypt-villus axis[11,20]. In the current study, we found that I/R, following occlusion of the SMA, led to statistically significant increment of apoptosis in undifferentiated epithelial cells located in the proliferative compartment of the adult rat's small intestine, as well as in differentiated epithelial cells distributed in the villus at 2-12 h after reperfusion. The most apoptotic rates in the upper portion of the villus are the region, which is farthest from submucosal vessels that supply blood to the mesenchymal core of the villus. The asymmetric distribution of apoptosis may reflect the fact that hypoxia and deficient nutrient generated by SMA occlusion are greater in epithelial cells located in the upper villus compared with crypts. However, it is also possible that the signaling pathways that mediate the induction differ qualitatively or quantitatively between undifferentiated proliferating cells in the crypt and postmitotic differentiated cells in the upper villus. The actual mechanism needs to be deeply explored. Our studies displayed that administration of exogenous aFGF produced an approximately 1.5-fold reduction in the number of TUNEL positive cells that appear following transient SMA occlusion in proliferating undifferentiated crypt epithelial lineage as well as their postmitotic differentiated villus descendants. These results are compatible with the notion that exogenous aFGF might protect from intestinal injury caused by I/R insult through an identical mechanism in villus and crypt epithelial cells.

D(-)-lactate is the stereoisomer of mammalian L(+)-lactate. Mammalian tissue cannot produce D(-)-lactate and can only slowly metabolize it. It is a strict product of bacterial fermentation. Since intestinal I/R injury can cause mucosal injury and subsequent bacterial proliferation, D(-)-lactate releasing from the gut into the host blood circulation, the change of plasma D(-)-lactate level was used as a predictor of intestinal I/R injury[21,22]. In the present study, it was shown that plasma D(-)-lactate levels began to increase in rat's intestinal villus subjected to 45 min of ischemia followed by 30 min of reperfusion, maximal at 12 h after reperfusion. In the aFGF treated group, it was significantly decreased during 2-12 h after reperfusion compared with normal saline treated group, which was corroborated with the data obtained by the TUNEL method, indicating that aFGF exerts a positive protective effect on the mucosal barrier and decreases the intestinal permeability.

Our study also found that apoptosis occurred at 2 h post-reperfusion fδllowed by a return to baseline values by 24 h suggestsing that I/R induction of intestinal apoptosis and mucosal recovery were rapid processes. The reason for this interesting kinetics of induction of mucosal cell death and restoration was unclear. PCNA is a significant cell-cycle regulated nuclear protein for DNA-polymerase. PCNA-labeled nuclei identify cells in the late G1 and early S phases of the cell cycle, as well as cells undergoing DNA repair[23,24]. Our current study demonstrated that the expressing levels of PCNA at 2-12 h after reperfusion were significantly higher than the levels of C control in both crypt and villus epithelial cells. These findings indicated that the repair process of small intestinal mucosa was initiated by I/R and cellular regeneration continued for 12 h following reperfusion. At the same time, the PCNA protein was mainly located in the nuclei of proliferative cells in crypts. Administration of exogenous aFGF could induce protein expression of PCNA in mucosal cells, especially in crypt epithelial cells. At 2-12 h after reperfusion, PCNA was strongly expressed both in undifferentiated crypt epithelial cells as well as differentiated villus cells in comparison with normal saline treated group. These results suggest that aFGF treatment could inhibit cellular apoptosis of mucosal cells and induced cell proliferation, especially cells in crypt, to accelerate regeneration and repair of small intestinal mucosa after ischemia-reperfusion insult. Ischemia of the adult human small intestine could result in high mortality[25]. Because administration of exogenous aFGF can ameliorate the reperfusion injury that accompanies intestinal transplantation or the epithelial wound brought about by sepsis, interventions that elevate aFGF levels or enhance its activity may be useful for reducing the damage produced by ischemia-reperfusion insult.

In conclusion, the present study provided the primary evidence that the protective effects of aFGF against ischemia/reperfusion in rat's intestinal villus might be partially due to its abilities to inhibit ischemia/reperfusion-induced apoptosis and to promote crypt and villus epithelial cell proliferation. The precise mechanism for the attenuation of intestinal ischemia/reperfusion injury and acceleration of mucosal regeneration afforded by aFGF

requires further investigation.

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