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• *BASIC RESEARCH* •

Exogenous acid fibroblast growth factor inhibits ischemia-reperfusion-induced damage in intestinal epithelium via regulating P53 and P21WAF-1 expression

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Abstract

AIM: To detect the effect of acid fibroblast growth factor (aFGF) on P53 and P21WAF-1 expression in rat intestine after ischemia-reperfusion (I-R) injury in order to explore the protective mechanisms of aFGF.

METHODS: Male rats were randomly divided into four groups, namely intestinal ischemia-reperfusion group (R), aFGF treatment group (A), intestinal ischemia group (I), and sham-operated control group (C). In group I, the animals were killed after 45 min of superior mesenteric artery (SMA) occlusion. In groups R and A, the rats sustained for 45 min of SMA occlusion and were treated with normal saline (0.15 mL) and aFGF (20 μg/kg, 0.15 mL), then sustained at various times for up to 48 h after reperfusion. In group C, SMA was separated, but without occlusion. Apoptosis in intestinal villi was determined with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling technique (TUNEL). Intestinal tissue samples were taken not only for RT-PCR to detect P53 and P21WAF-1 gene expression, but also for immunohistochemical analysis to detect P53 and P21WAF-1 protein expression and distribution.

RESULTS: In histopathological study, ameliorated intestinal structures were observed at 2, 6, and 12 h after reperfusion in A group compared to R group. The apoptotic rates were (41.17±3.49)%, (42.83±5.23)%, and (53.33±6.92)% at 2, 6, and 12 h after reperfusion, respectively in A group, which were apparently lower than those in R group at their matched time points (50.67±6.95)%, (54.17±7.86)%, and (64.33±6.47)%, respectively, (P<0.05)). The protein contents of P53 and P21WAF-1 were both significantly decreased in A group compared to R group (P<0.05) at 2-12 h after reperfusion, while the mRNA levels of P53 and P21WAF-1 in A group were obviously lower than those in R group at 6-12 h after reperfusion (P<0.05).

CONCLUSION: P53 and P21WAF-1 protein accumulations are associated with intestinal barrier injury induced by I-R insult, while intravenous aFGF can alleviate apoptosis of rat intestinal cells by inhibiting P53 and P21WAF-1 protein expression.

Key words: Acid fibroblast growth factor; Ischemia; Reperfusion; P53 gene; P21WAF-1 gene

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INTRODUCTION

Intestinal ischemia-reperfusion (I-R) injury is characterized histologically by inflammation, villus abscission, and mucosal epithelial cell apoptosis[1]. Agents can modulate or prevent apoptosis after I-R[2-6]. Though the mechanisms of action are diverse, all these agents ultimately show their potent antiapoptotic properties that account, at least in part, for their protective effects. Accumulation of P53 protein, which is well known as a tumor suppressor gene product, plays a central role as the initiator of the intrinsic apoptotic cascade triggered by a wide variety of insults[7-10]. In addition, a role of P53 in regulating the extrinsic receptor-mediated apoptotic pathway has also been reported[11]. P21WAF-1, which is a downstream mediator of P53 function, plays a key role in determining the ultimate sensitivity of cells to myriad stimuli and insults that induce apoptosis[12-14]. Thus, P53 and P21WAF-1 are poised as the ideal candidates for mediating apoptosis after I-R, a setting

where many insults coexist.

Acid fibroblast growth factor (aFGF) is a mitogen *in vitro* for most of the ectoderm- and mesoderm-derived cell lines. In addition, this factor shows a wide range of endocrine-like activities[15,16]. As a multiple function growth factor, aFGF is involved in embryo development and tissue repair[17,18]. Previous studies have shown that intravenous administration of exogenous aFGF could improve the physiological functions of intestine after I-R injury[19,20]. Since the mechanism by which aFGF inhibits apoptosis is unknown, we investigated the effects of aFGF on gene expression and protein contents of P53 and P21WAF-1 underlying the protective mechanisms of aFGF against intestinal 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 Sciences, Beijing) were used in this study. Animals were housed in wire-bottomed cages placed in a room illuminated from 08:00 to 20:00 (12:12-h light-dark cycle) and maintained at 21±1 ℃. Rats were allowed free access to water and food. The animals were anesthetized with 3% sodium pentobarbital (40 mg/kg) and underwent laparotomy. The superior mesenteric artery (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 four groups, namely intestinal ischemia-reperfusion group (R), intestinal ischemia group (I), reconstructive aFGF treatment group (A) and sham-operated control group (C). In group I, the animals were killed after 45 min of SMA occlusion. In groups R and A, the rats sustained for 45 min of SMA occlusion and were treated with 0.15 mL normal saline and 0.15 mL saline plus aFGF (20 μg/kg) injected from tail vein, then sustained for 15, 30 min, 1, 2, 6, 12, 24, and 48 h of reperfusion respectively. In C group, SMA was separated, but without occlusion, and samples were taken after exposure of SMA for 45 min. In groups R and A, rats were killed at different time points after reperfusion, and intestinal tissue biopsies were taken. Six specimens of the intestine were obtained at each time point from six rats. When any rat died during the study, additional rats were used until six specimens could be obtained from six rats at each time point. A small part of the intestine specimen was fixed with 10% neutral buffered formalin for immunohistochemical detections of intestinal epithelial apoptosis, and protein expression of P53 and P21WAF-1. The remaining tissue samples were placed in liquid nitrogen for RT-PCR to detect P53 and P21WAF-1 gene expression.

Histopathological study

Formalin-fixed, paraffin-embedded intestinal samples were cut into 5-μm-thick sections, deparaffinized in xylene, rehydrated in graded ethanol, and then stained with hematoxylin-eosin (HE) for histological observation under light microscope.

In situ detection of cell death

The apoptotic cells in intestinal tissues were detected by 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 and then incubated with 20 μg/mL proteinase K for 15 min. Seventy-five microliters of equilibration buffer was applied directly onto the specimens for 10 min, 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 and then covered with 55 μL of anti-digoxigenin peroxidase and incubated for 30 min. 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× microscope. Sixty intestinal villi per time point were required for counting, and then the apoptotic ratios were calculated and analyzed.

Immunohistochemistry

Immunostaining for proteins of P53 and P21WAF-1 was performed in paraffin sections with a high-temperature antigen-unmasking method in citrate buffer and ABC peroxidase, using P21WAF-1 monoclonal mouse antibody (Santa Cruz Cor, sc-6246) and P53 polyclonal rabbit antibody (Santa Cruz Cor, sc-6243) against antigens (1:100 in PBS). Tissues were fixed overnight in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Five-micrometer thick sections 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. Tissue sections were then incubated for 60 min with biotinylated secondary antibody. After being washed in PBS, the sections were exposed to acidin-biotin complex for 60 min, reacted with 0.05% (wt/vol) DAB in 50 mmol/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× microscope. Sixty intestinal villi per time point were required for counting, and then the ratio of positive cells was calculated and analyzed.

RNA extraction and RT-PCR analysis

Tissue total RNA was extracted using TRIzol reagent

(Gibco BRL, USA). RNA was serially diluted with water containing 1 unit RNase inhibitor per μL and 3 mmol/L dithiothreitol (DTT). One microliter RNA, 1 μL oligo (dT12-18), 1 μL avian myeloblastosis virus reverse transcriptase (AMV-RT), 2 μL 10 mmol/L deoxynucleoside triphosphate (dNTP), 2 μL 0.1 mol/L DTT, 4 μL 5× buffer, and sterilized distilled water up to a total volume of 20 μL were incubated at 37 ℃ for 60 min. Subsequently, 2 μL of each reaction product was amplified in 50 μL of a PCR mixture. Then 29 cycles were performed with a Perkin-Elmer Cetus/DNA thermal cycler (Takara Shuzo Co., Tokyo, Japan) at 94 ℃ for 1 min, at 50 ℃ for 1 min, at 72 ℃for 1 min, and then at 72 ℃ for 10 min at the end of the procedure. In this study, β-actin, which is ubiquitously expressed, was used as a positive control in a pilot study before formal experimentation, and PCR reaction for each primer set was repeated four times to verify the reproducibility of results. After PCR, 5-μL sample aliquots was electrophoresed on a 2% agarose gel for 30 min, stained with ethidium bromide and photographed. Densitometry was done with a Bechman densitometer. The level of gene transcription was expressed as the ratio of gray density of the gene to β-actin.

Statistical analysis

All values were expressed as mean±SD. The statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Student's and Newman-Keuls multiple comparison tests. $P<0.05$ was considered statistically significant.

RESULTS

Histopathological findings

After the SMA was clamped near its origin from the aorta, the entire small intestine showed a dark purple, cyanotic color within 45 min. The histological evaluation revealed that damage to the small intestine in I group was small, with slightly edematous villus tips and intact crypts just after the ischemic period. Two hours after reperfusion, partial loss of the mucosa could be observed. During 6-12 h after reperfusion, the damage of intestinal epithelial cells, hemorrhage and apoptosis could be found accompanied with inflammatory cells infiltrated into the intestinal wall, and the crypt-villus structure was seriously spoiled. In the period of 24-48 h after reperfusion, the mucosal integrity was partially restored. Histological structure of the intestinal mucosa was markedly improved after administration of aFGF. The protective function of aFGF on intestinal mucosa was very effective 2-12 h after reperfusion. The structures of crypt and villus were both guarded, with less damage of intestinal mucosa in A group compared to R group.

Change of cellular apoptotic rates

To quantify the extent of apoptosis after ischemia and reperfusion, TUNEL reaction was performed in serial sections prepared from the middle quarter of the small intestine (jejunum). The time, when the animals were killed after a 45-min SMA occlusion followed by reperfusion, varied to further define the apoptotic response. Statistically significant increase in TUNEL-positive cells was not detectable until 1 h after reperfusion and reached its peak at 12 h. The cellular apoptotic rate in intestinal mucosa at 12 h after reperfusion was 3.3 times of that in C group. After reperfusion for 24 and 48 h, the mucosal apoptotic rates were restored to the level of C group. Administration of aFGF resulted in statistically significant decrease of the apoptotic rates compared to R group 2-12 h after reperfusion ($P<0.05$). No statistically significant decrease of apoptotic rates was observed at 24 and 48 h after reperfusion (Table 1).

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

Groups	R group	A group
C group	19.67±3.50	19.67±3.50
I group	27.67±9.63	27.67±9.63
15 min after reperfusion	29.50±5.61	25.17±6.43
30 min after reperfusion	28.00±7.02	26.00±4.86
1 h after reperfusion	34.67±5.47[a]	29.83±7.08
2 h after reperfusion	50.67±6.95[a]	41.17±3.49[ac]
6 h after reperfusion	54.17±7.86[a]	42.83±5.23[ac]
12 h after reperfusion	64.33±6.47[a]	53.33±6.92[ac]
24 h after reperfusion	28.50±5.47	23.33±3.83
48 h after reperfusion	26.00±5.76	22.00±4.60

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

Expression characteristics of P53 and P21WAF-1 proteins

Quantitative immunohistochemical results for P53 and P21WAF-1 proteins are summarized in Table 2. Protein expression levels of P53 were weaker in the sham-operated intestinal and ischemic tissues, and positive particles were mainly located in the epithelial cells of the upper part of villi. However, the positive cellular rates elevated with the increment of duration after reperfusion injury. In the period of 1-12 h after reperfusion, P53 protein was expressed at a dramatically higher level in comparison to C group ($P<0.05$) and the maximum level was 2.1-fold of C group at 6 h after reperfusion. The positive signals of P53 were mostly distributed in the nuclei and cytoplasm of epithelial cells of villi and crypts. At 24-48 h after reperfusion, the positive cellular rates were not substantially changed compared to C group. Treatment with aFGF could apparently inhibit the protein contents of P53 in intestinal mucosal epithelial cells 2-12 h after reperfusion in comparison to R group at different matched time points ($P<0.05$, Figures 1A-1C). The levels of P21WAF-1 protein were also significantly increased 2-12 h after reperfusion with a peak at 12 h after reperfusion (1.5-fold of C group, $P<0.05$). The positive particles of P21WAF-1 were mainly localized in the cytoplasm and nuclei of intestinal epithelial cells (Figures 1D-1F). By 24-48 h after I-R, the P21WAF-1 levels tended to normalize back to baseline of C group ($P>0.05$). Compared to the saline-treated group, the positive cellular rates of P21WAF-1 were significantly

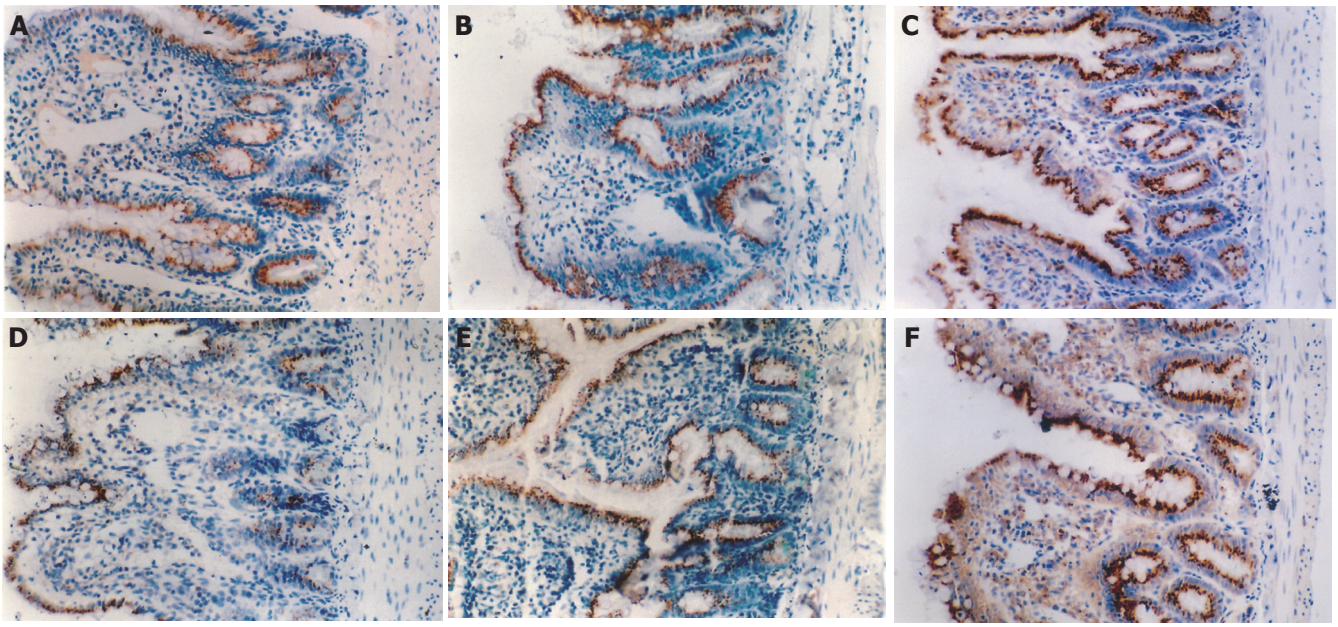

Figure 1 Expression of P53 and P21^{WAF-1} protein in C group (**A** and **D**, respectively), A group (**B** and **E**, respectively) and R group (**C** and **F**, respectively).

Table 2 Effect of aFGF on protein expression of P53 and P21WAF-1 in intestinal mucosa after ischemia-reperfusion (n = 6, mean±SD, %)

Groups	P53		P21WAF-1	
	R group	A group	R group	A group
C group	23.67±4.55	23.67±4.55	32.50±3.94	32.50±3.94
I group	29.17±4.45	29.17±4.45	35.83±4.83	35.83±4.83
15 min after reperfusion	27.00±3.90	23.83±5.08	34.67±4.76	31.50±4.37
30 min after reperfusion	30.67±3.98	26.67±3.78	36.17±3.31	37.00±4.86
1 h after reperfusion	33.17±3.19[a]	32.50±3.27[a]	39.83±4.58	37.83±4.36
2 h after reperfusion	40.33±3.50[a]	33.83±5.04[a,c]	49.33±4.18[a]	41.83±6.65[a,c]
6 h after reperfusion	50.50±4.23[a]	42.67±3.88[a,c]	53.00±4.39[a]	44.67±4.46[a,c]
12 h after reperfusion	45.67±5.65[a]	37.50±4.81[a,c]	57.50±3.62[a]	49.33±3.78[a,c]
24 h after reperfusion	30.00±6.13	25.33±3.33	34.50±6.02	30.00±3.79
48 h after reperfusion	28.17±8.89	25.67±4.32	33.83±7.31	30.67±4.18

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

Table 3 Effect of aFGF on mRNA contents of P53 and P21WAF-1 genes in intestinal mucosa after ischemia-reperfusion (n = 6, mean±SD, %)

Groups	P53		P21WAF-1	
	R group	A group	R group	A group
C group	23.5±6.0	22.4±2.5	26.7±2.0	24.6±4.1
I group	38.9±5.1[a]	34.8±4.2[a]	32.6±2.0[a]	31.7±2.0[a]
15 min after reperfusion	53.9±3.9[a]	45.8±10.7[a]	33.4±2.3[a]	24.0±3.4[c]
30 min after reperfusion	34.0±4.4[a]	41.9±5.2[a,c]	23.3±4.2	22.9±2.0
1 h after reperfusion	48.5±3.2[a]	44.7±5.4[a]	24.8±2.3	23.8±3.2
2 h after reperfusion	46.7±5.4[a]	41.3±6.3[a]	26.5±2.7	24.9±3.0
6 h after reperfusion	48.8±5.2[a]	38.3±4.7[a,c]	30.8±3.0[a]	20.9±2.6[c]
12 h after reperfusion	51.0±4.5[a]	34.1±4.8[a,c]	32.7±1.6[a]	21.9±2.8[c]
24 h after reperfusion	55.3±7.3[a]	56.9±5.3[a]	28.8±3.1	28.5±5.0
48 h after reperfusion	56.4±8.3[a]	61.0±4.0[a]	29.0±2.3	28.9±3.8

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

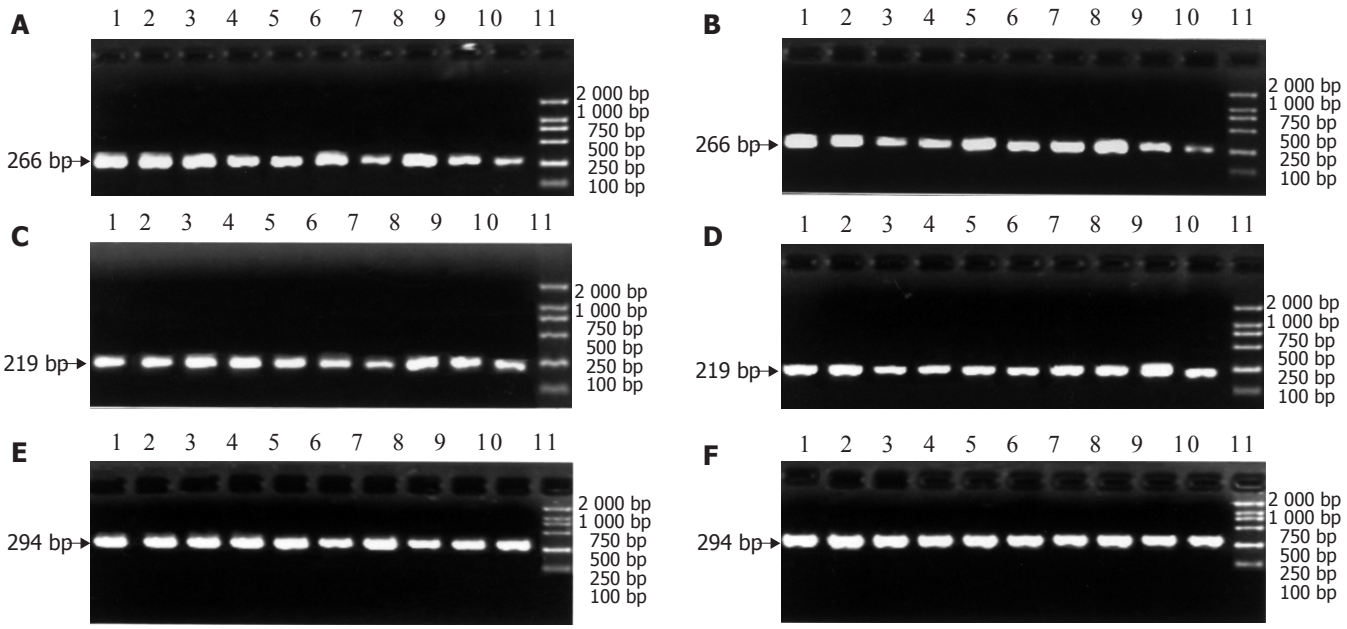

Figure 2 Expression of P53, $P21^{WAF-1}$, and β-actin genes in normal saline-treated (**A**, **C**, **E**, respectively) and aFGF treated (**B**, **D**, **F**, respectively) rat intestinal villi. Bar indicates the size of RT-PCR cDNA products. Lanes 1-8: 48, 24, 12, 6, 2, 1 h, and 30, 15 min after reperfusion; lane 9: ischemia group; lane 10: sham-operated control group; lane 11: DL 2000 marker.

lower 2 to 12 h after reperfusion in aFGF-treated group ($P<0.05$, Table 2).

Expression characteristics of P53 and P21WAF-1mRNA

We investigated the gene expression of P53 and P21WAF-1 in differentially treated intestinal villi through RT-PCR analysis (Table 3). The P53 gene amplification product was composed of 266 bp (Figures 2A-2C). Expression of this gene was remarkably and rapidly increased in intestinal mucosa after ischemia during the whole period of reperfusion. After aFGF administration, the mRNA level of P53 in villus cells was lower than that in normal saline-treated group. Especially at 30 min, 6 and 12 h after reperfusion, the discrepancy of P53 expression levels between the two groups was apparent ($P<0.05$, Table 3). Figures 2D-2F show that the length of RT-PCR products of P21WAF-1 was 219 bp. The P21WAF-1 gene was expressed at a pronounced high level in villi compared to sham-operated group at 15 min, 6 and 12 h after reperfusion ($P<0.05$). After treatment with aFGF, although P21WAF-1 gene expression was not substantially decreased after reperfusion in A group compared to C group, the content of P21WAF-1 gene transcript were markedly reduced at 15 min, 6 and 12 h after reperfusion in A group compared to R group ($P<0.05$, Table 3).

DISCUSSION

The major clinical disorders involving gastrointestinal circulation are hemorrhage and ischemia. It is well recognized that the small intestine is extremely sensitive to the deleterious effects of I-R, and it has been clearly demonstrated that I-R causes both mucosal and vascular injury within the small intestine[21,22]. Intestinal I-R injury may also cause release of bacteria and toxins from the gut into the host blood circulation and changes of inflammatory factors, cytokines, and growth factors, resulting in damage to the intestinal barrier. In the current study, we found that I-R, following occlusion of the SMA, induced apoptosis in the intestinal mucosal cells. Results of TUNEL method displayed that the apoptotic rate increased during ischemia and peaked at 12 h after reperfusion. The locations of apoptotic cells were extended from villus tip in sham-operated rats to the whole structure of mucosa in rats insulted by I-R. The quantification of apoptosis was corroborated with histological examination using HE staining. It was also found that administration of exogenous aFGF could reduce the intestinal injury caused by I-R insult. The antiapoptotic effect of aFGF was achieved by inhibiting the expression of some apoptosis-related genes. Previous experimental data showed that the change of expression of apoptosis-related genes played a pivotal role in alleviating cardiac, myocardial[23], cerebral[24,25], muscular[26], cutaneous[27], and adrenal cortex[28] apoptosis induced by I-R, which was in agreement with our results that I-R promoted P53 and P21WAF-1 expression.

After 45 min of ischemia, although gene expression of P53 was quickly increased, and lasted for the whole period after reperfusion, P21WAF-1 gene was only strongly expressed at 15 min, 6 h, and 12 h after reperfusion. The difference of time in the kinetics between these two genes may indicate that P21WAF-1 gene transcription might be activated in a P53-independent manner. Moreover, although protein levels of P53 and P21WAF-1 accumulated after reperfusion, there was a time lag in the

onset of elevation and the peak time point between these two proteins. These results indicate that the P21WAF-1 translation is activated by the elevated P53 protein contents. In this current study, we also found that the kinetics between the levels of P21WAF-1 and P53 protein expression and the apoptotic rate were similar, suggesting that protein levels of P21WAF-1 and P53 might be related to cell apoptosis. When severe histological damage of intestinal villi 2-12 h after reperfusion is considered, DNA damage in the intestinal cells cannot be repaired, resulting in cell apoptosis. Our study also found that the rate of apoptosis in intestinal villi insulted by I-R was significantly decreased by aFGF administration. aFGF could inhibit the increments of P21WAF-1 and P53 protein expression 2-12 h after reperfusion in comparison to normal saline treatment, suggesting that the decrement of P21WAF-1 and P53 protein contents caused by aFGF might be one of the mechanisms attenuating ischemia-reperfusion-induced apoptosis.

Studies have demonstrated that the activated neutrophils and oxygen free radicals produced in ischemic tissue during reperfusion play an important role in developing the injury of the intestine[26,28]. Free radicals are produced mainly by the activated neutrophils and xanthine dehydrogenase/xanthine oxidase enzyme system after reperfusion, but the free radicals produced from the neutrophils play a more important role than xanthine oxidase in mediating tissue-destroying events[29]. Our histopathological study showed that leukocyte sequestration into the villi was evident 6-12 h after reperfusion, and exogenous aFGF could alleviate leukocyte infiltration into intestinal villi. It is well known that free radicals cause DNA damage, and the series of cell reactions mediated by P53 might be investigated by free radicals produced during the reperfusion process. The protective mechanism of aFGF might inhibit P53 and P21WAF-1 protein translation by scavenging free radicals. The current study also demonstrated that apoptosis occurred 1 h after reperfusion and returned to baseline values after 24 h, suggesting that I-R-induced intestinal apoptosis and mucosal recovery is a rapid process. The mechanism underlying this interesting kinetics of induction of mucosal cell apoptosis and restoration is unclear. A time-dependent increment in protein expression of apoptosis-promoting factors including P53 and P21WAF-1 during ischemia and early phases of reperfusion, and decrement with prolonged reperfusion might be an alternative cause. Therefore, the protective effect of aFGF against intestinal I-R insult might be not only associated with inhibiting epithelial cell apoptosis but also related to inducing mucosal cell restoration.

In conclusion, the protective effects of aFGF against I-R in rat intestinal villi might be partially due to its ability to inhibit I-R-induced apoptosis. aFGF exerts its antiapoptotic effect via regulating P53 and P21WAF-1gene transcription and translation. The precise mechanisms of aFGF underlying the inhibition of intestinal I-R injury and attenuation of apoptosis need further investigation.

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