Intravenous acid fibroblast growth factor protects intestinal mucosal cells against ischemia-reperfusion injury via regulating Bcl-2/Bax expression

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AIM: To detect the effect of acid fibroblast growth factor (aFGF) on apoptosis and gene expression of bax and bcl-2 gene in rat intestine after ischemia/reperfusion (I/R) injury, and to explore the protective mechanisms of aFGF.

METHODS: One hundred and eight 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). In group I, the animals were killed after 45 min of superior mesenteric artery (SMA) occlusion, while in groups R and A, the rats sustained 45 min of SMA occlusion and were then treated with normal saline and aFGF, respectively, sustained 15 min, 30 min, 1, 2, 6, 12, 24, or 48 h of reperfusion, respectively. In group C, SMA was separated, but without occlusion. Apoptosis in intestinal villus was determined with terminal deoxynucleotidyl transferase mediated dUTP-biotin nick-end labeling technique (TUNEL). Intestinal tissue samples were taken not only for detection of bax and bcl-2 gene expression by RT-PCR, but also for detection of bax and bcl-2 protein expression and distribution by immunohistochemical analysis.

RESULTS: The rat survival rates in aFGF treated group were higher than group R (P<0.05) and the improvement of intestinal histological structures was observed at 2, 6, and 12 h after the reperfusion in group A compared with group R. 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 group A, apparently less than those of group R at matched time points (50.67±6.95, 54.17±7.86, 64.33±6.47, respectively) (P<0.05). The bax gene transcription and translation were significantly decreased in group A vs group R, while mRNA and protein contents of Bcl-2 in group A were obviously higher than those in group R during 2-12 h period after reperfusion.

CONCLUSION: The changes in histological structure and the increment of apoptotic rate indicated that the intestinal barrier was damaged after intestinal I/R injury, whilst intravenous aFGF could alleviate apoptosis induced by ischemia and reperfusion in rat intestinal tissues, in which genes of bax and bcl-2 might play important roles.

Key words: Acid fibroblast growth; Ischemia; Reperfusion; Bcl-2 gene; Bax gene

INTRODUCTION
aFGF belongs to a family of at least 20 related growth factors that bind to varying extents to four different cell surface receptors and their splicing variants. aFGF and basic FGF(bFGF) are the prototypic members of the family, and share a similar broad spectrum of biological activities. These two growth factors are mitogens in vitro for most of the ectodermal- and mesodermal-derived cell lines. In addition, these proteins show a wide range of endocrine-like activities[1-4]. Previous studies have shown that intravenous administration of exogenous bFGF could improve the physiological functions of intestine after I/R injury[5-8]. However, the protective mechanisms of aFGF on intestinal I/R injury remain unknown.

Apoptosis, a form of death characterized by cell shrinkage, plasma membrane blebbing, chromatin condensation and genomic DNA fragmentation, is essential for development and maintenance of tissue homeostasis[6,7]. On the other hand, apoptosis has been implicated in many diseases such as intestinal ischemic and reperfusion insult. I/R induced apoptosis in the jejunum and ileum[8]. However, little investigation has been conducted to determine whether the protective effect afforded...
by aFGF relates to reduction in apoptosis during ischemia/reperfusion. The objective of this study was therefore to determine whether aFGF could protect the rat intestinal mucosa against ischemia/reperfusion-induced apoptosis. Since bcl-2 family of proteins plays a major role in determining the ultimate sensitivity or resistance of cells to myriad stimuli and insults that induce apoptosis\(^ {9-11}\), we also examined the effects of aFGF on gene expression of bcl-2 family underlying the protective mechanisms of aFGF on intestinal ischemia injury.

**MATERIALS AND METHODS**

**Animal model and experimental design**

One hundred and eight healthy male Wistar rats weighing 220±20 g (Animal Center, Chinese 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 to 20:00 (12:12 h light-dark cycle) and maintained at (21±1) °C. Rats were allowed free access to water and chow ad libitum. After the animals received anesthesia by 3% sodium pentobarbital (40 mg/kg), a laparotomy was performed. 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 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) (\(n=48\)). 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 15, 30 min, 1, 2, 6, 12, 24, or 48 h of reperfusion, respectively. In group C, 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. A small piece of tissue sample was fixed with 10% neutral buffered formalin for immunohistochemical detection of intestinal epithelial apoptosis, and protein expression of bax and bcl-2. The rest of tissue samples were placed in liquid nitrogen for detection of bax and bcl-2 gene expression by RT-PCR.

**Histological staining**

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-eosine (HE) for histological observation under light microscope. In situ detection of cell death

The apoptotic cells in intestinal tissues were detected with the terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-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 equilibration buffer were applied directly onto the specimens for 10 min at room temperature, followed by 55 µL of TdT enzyme and then at 37 °C for 1 h. The reaction was terminated by transferring the slides to prewarmed stop/wash buffer for 30 min at 37 °C. 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.

**Immunohistochemistry**

Immunostaining for bax and bcl-2 proteins was performed on paraffin sections with a high-temperature antigen-unmasking method in citrate buffer and ABC peroxidase, using monoclonal mouse antibodies against antigens (Santa Cruz Co., 1:100 in PBS), a goat anti-mouse secondary antibody (Santa Cruz Co., 1:100) and DAB substrate kit (Santa Cruz Co.). Sections of 5 µm thickness were cut and mounted onto slides coated with 3-triethoxysilylpropylamide. They were rehydrated and submerged in 3% hydrogen peroxide for 10 min, washed in PBS for 5 min, then blocked with 5% normal swine serum in PBS for 30 min at room temperature, followed by incubation with primary antibodies at a concentration of 5 µg/mL (diluted in PBS containing 5% swine serum) overnight at 4 °C. Control slides were incubated with PBS without primary antibodies. Tissue sections were then washed in PBS and incubated for 60 min at room temperature with biotinylated secondary antibody and PBS. After washing in PBS, the sections were exposed to acidin-biotin complex for 60 min at room temperature and again washed in PBS. The sections were reacted with 0.5 mL/L DAB in 50 mmol/L Tris-HCl (pH 7.4), with 1 mL/L hydrogen peroxide for 5 min and counte-rstained with hematoxylin. The results of positive staining cells and their distribution were observed under a microscope of 400 magnification. Fifty intestinal villi were required for counting, and then the ratio of positive cells were calculated and analyzed.

**RNA extraction and RT-PCR analysis**

Total RNA was extracted using TRIzol reagent (Gibco BRL, USA), and then was serially diluted with water pretreated with diethylpyrocarbonate containing one unit RNase inhibitor per µL and 3 mmol/L dithiothreitol. RNA 1 µL, oligo (dT\(_{12\text{-}18}\)) 1 µL, avian myeloblastosis virus reverse transcriptase (AMV-RT) 1 µL, 10 mmol/L deoxynucleoside triphosphate (dNTP) 2 µL, 0.1 mol/L DTT 2 µL, 5× buffer 4 µL, and sterilized distilled water up to a total volume of 20 µL were incubated at 37 °C for 60 min. After reverse transcription, samples were heated at 95 °C for 5 min to denature the AMV-RT and stored at -20 °C for PCR. 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 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and then at 72 °C for 10 min at the end of the procedure. Table 1 shows the synthesized oligonucleotide primers used for RT-PCR for genes of bax and bcl-2. In this study, β-actin, which is ubiquitously expressed, was used as a positive control in a pilot study before formal experimentation, and PCR reactions for each primer set were repeated four times to verify reproducibility of results. After PCR, 5 µL sample aliquots were 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 expression was expressed as the ratio of the gray density of the objective gene over the gray density of β-actin.

**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 was considered as statistically significant.

**RESULTS**

**Change of morphological appearance**

The histological evaluations revealed that damage to the small intestine in the 45-min ischemia group was small, with slightly edematous villus tips just after the ischemic period, while the partial loss of the mucosa could be observed at 2 h after reperfusion. During 6-12 h after reperfusion, the damage of intestinal epithelial cells, hemorrhage and necrosis could be found, accompanied by inflammatory cell infiltration into the intestinal wall. In the period of 24-48 h after reperfusion, the mucosal integrity was partially restored. Histological structure of the intestinal mucosa after reperfusion for 12 h was 3.3 times that in group C. After reperfusion for 24 and 48 h, the mucosal apoptotic rates were restored to the level in group C. Administration of aFGF produced statistically significant decreases in the apoptotic rates compared with group R during 2-12 h after reperfusion (*P<0.05). No significant decreases in apoptosis were observed at 24 and 48 h after reperfusion (Table 2).

**Expression characteristics of bax and bcl-2 proteins**

Quantitative immunohistochemical results for bax protein and bcl-2 protein were evaluated in Table 3. Protein

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**Table 1** Oligonucleotide primers for RT-PCR detection of bax, bcl-2 and β-actin mRNA transcripts

| Genes     | Upper primers | Lower primers            |
|-----------|---------------|--------------------------|
| bax       | 5'-AGGCTTTTCCGGACTGCCAGC-3' | 5'-CCCGGGAGGAAGTCCAATGTCC-3' |
| bcl-2     | 5'-GACTGCGGAGATGTCAGCAG-3' | 5'-GTGGCAGGTGGCCGTCAGG-3'   |
| β-actin   | 5'-AGCCATGTACGTAGCCATCC-3' | 5'-GCCATCTCTTGTGAAGTC-3'    |

**Table 2** Effect of aFGF on the apoptotic rates in intestinal mucosa after ischemia-reperfusion insult (*n=6, mean±SD, %)

| Groups | Group R | Group A |
|--------|---------|---------|
| Group C | 19.67±3.39 | 19.67±3.39 |
| Group I | 27.67±4.63 | 27.67±4.63 |
| 15 min after reperfusion | 29.50±5.61 | 25.17±6.43 |
| 30 min after reperfusion | 31.00±5.02 | 26.00±4.86 |
| 1 h after reperfusion | 34.67±5.47 | 32.82±7.08 |
| 2 h after reperfusion | 50.67±6.95 | 41.17±4.94 |
| 6 h after reperfusion | 54.17±6.86 | 42.83±5.23 |
| 12 h after reperfusion | 64.33±6.47 | 53.33±6.92 |
| 24 h after reperfusion | 28.50±5.47 | 23.33±3.83 |
| 48 h after reperfusion | 26.00±5.76 | 22.00±4.60 |

*P<0.05 vs group C; *P<0.05 vs group R at matched time points.

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**Table 3** Effect of aFGF on protein contents of bax and bcl-2 in intestinal villus after ischemia-reperfusion (*n=6, mean±SD, %)

| Groups | Bax protein | Bcl-2 protein |
|--------|-------------|---------------|
| Group C | 19.83±3.66 | 35.33±4.59 |
| Group I | 23.83±4.65 | 28.17±4.12 |
| 15 min after reperfusion | 25.00±5.93 | 29.67±5.10 |
| 30 min after reperfusion | 28.00±9.23 | 20.67±5.49 |
| 1 h after reperfusion | 37.83±4.75 | 24.50±6.56 |
| 2 h after reperfusion | 47.67±7.26 | 20.67±7.15 |
| 6 h after reperfusion | 54.67±5.28 | 18.83±5.35 |
| 12 h after reperfusion | 46.33±4.27 | 19.50±4.37 |
| 24 h after reperfusion | 29.00±4.43 | 28.67±2.94 |
| 48 h after reperfusion | 26.17±3.49 | 29.00±3.74 |

*P<0.05 vs group C; *P<0.05 vs group R at matched time points.
expression of bax was weak in the sham-operated intestinal tissues and ischemic tissues. However, the positive cellular rate elevated with the increment of duration after reperfusion injury. In the period of 2-12 h after reperfusion, bax was expressed at a dramatically higher level in comparison with group C (P<0.05) and positive signals of bax were distributed in the whole structure of villus, including mucosa, submucosa, lamina propria and myometrium. At 24 h after reperfusion, the positive cellular rate was not substantially changed compared with group C. Treatment of aFGF could apparently inhibit the protein expression of Bax in intestinal mucosa during 2-12 h after reperfusion in comparison with group R at different matched times (P<0.05) (Figure 1). There was a negative correlation between positive expression levels and the intensities of bax and bcl-2. Bcl-2 was strongly expressed in the sham-operated intestinal tissues and positive particles of bcl-2 were mainly localized in the cytoplasm and nuclei of villus cells and in the nuclei of crypt cells (Figure 2). bcl-2 expression level in intestinal mucosa decreased with increasing reperfusion time, and the positive cellular rate was restored to the level of sham-operated tissues at 48 h after reperfusion. Compared with the normal saline treated group, the positive cellular rates of bcl-2 were significantly higher during the period from 2 to 12 h after reperfusion in aFGF treated group (P<0.05).

Expression characteristics of bax and bcl-2 mRNA

We investigated gene expression of bax and bcl-2 in differentially treated intestinal villus through RT-PCR analysis (Table 4). Figure 3 shows bax gene amplification product in villus of differentially treated groups, which was composed of 168 bp, had a remarkable and rapid increment in intestinal villus after ischemia and reperfusion, peaked at 1 h after reperfusion, and then decreased to the levels similar to that of sham-operated group. After aFGF administration, the levels of bax in villous cells were apparently lower than that in normal saline treated group, especially at 15 min, 1, 2 and 6 h after reperfusion (P<0.05). The length of RT-PCR products of bcl-2 was 228 bp (Figure 4). Bcl-2 gene was expressed at a remarkably lower level in villus compared with sham-operated group after reperfusion. After treatment with aFGF, although bcl-2 gene was weakly expressed during the period from 15 min to 6 h after reperfusion versus group C, the level of bcl-2 gene expression returned to the level of sham-operated group at 12-48 h after reperfusion. In group A, the amounts of bcl-2 mRNA transcript were markedly elevated in comparison with group R at matched times after reperfusion (P<0.05), except for at 15 min after reperfusion (Table 4). The length of RT-PCR products of β-actin was 294 bp (Figure 5). As RT-PCR positive control, the quantity of β-actin mRNA was relatively the same in all intestinal villi analyzed (Figure 5).

DISCUSSION

It is well known that the intestinal mucosa is highly sensitive
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Table 4 Effect of aFGF on gene expression of bax and bcl-2 in intestinal villus after ischemia-reperfusion (n = 6, mean±SD, %)

| Groups                | Bax gene |            | Bcl-2 gene |            |
|-----------------------|----------|------------|------------|------------|
| Group C               |          |            |            |            |
| Group 1               |          |            |            |            |
| 15 min after Reperfusion |          |            |            |            |
| 30 min after Reperfusion |          |            |            |            |
| 1 h after Reperfusion  |          |            |            |            |
| 2 h after Reperfusion  |          |            |            |            |
| 6 h after Reperfusion  |          |            |            |            |
| 12 h after Reperfusion |          |            |            |            |
| 24 h after Reperfusion |          |            |            |            |
| 48 h after Reperfusion |          |            |            |            |

Figure 3 Expression of bax gene in normal saline treated (A) and aFGF treated (B) rat intestinal villus. Bar indicates the size of RT-PCR cDNA products. 1: 48 h after reperfusion, 2: 24 h after reperfusion, 3: 12 h after reperfusion, 4: 6 h after reperfusion, 5: 2 h after reperfusion, 6: 1 h after reperfusion, 7: 30 min after reperfusion, 8: 15 min after reperfusion, 9: ischemia group, 10: sham-operated control group, 11: DL2000 marker.

Figure 4 Expression of bax gene in normal saline treated (A) and aFGF treated (B) rat intestinal villus. Bar indicates the size of RT-PCR cDNA products. 1: 48 h after reperfusion, 2: 24 h after reperfusion, 3: 12 h after reperfusion, 4: 6 h after reperfusion, 5: 2 h after reperfusion, 6: 1 h after reperfusion, 7: 30 min after reperfusion, 8: 15 min after reperfusion, 9: ischemia group, 10: sham-operated control group, 11: DL2000 marker.

Figure 5 Expression of bcl-2 gene in normal saline treated (A) and aFGF treated (B) rat intestinal villus. Bar indicates the size of RT-PCR cDNA products. 1: 48 h after reperfusion, 2: 24 h after reperfusion, 3: 12 h after reperfusion, 4: 6 h after reperfusion, 5: 2 h after reperfusion, 6: 1 h after reperfusion, 7: 30 min after reperfusion, 8: 15 min after reperfusion, 9: ischemia group, 10: sham-operated control group, 11: DL2000 marker.

found that I/R, following occlusion of the SMA, induced apoptosis in the intestinal villus. Results of TUNEL examination displayed that the apoptotic rate increased during ischemia and was maximal at 12 h after reperfusion. The location of apoptotic bodies was extended from villus tip in sham-operated rats to the whole structure of villus, including mucosa, submucosa, lamina propria and myometrium in rat insulted by I/R. The quantification of apoptosis was corroborated with histological examination using HE: staining. We also found that administration of exogenous aFGF could reduce the intestinal injury caused by I/R insult. The antiapoptotic effect of aFGF was through regulation of expression of genes related to apoptosis. Previous experimental data showed that the change of gene expression of apoptosis-related genes played pivotal roles in alleviating cardiac, myocardial [12], cerebral [13,14], muscular [15], cutaneous [16] and adrenal cortex [17] apoptosis induced by I/R, which is in agreement with our results.

Apoptosis is governed by a number of regulatory genes mediated by apoptosis signals. Among them, the bcl-2 family of proteins constitutes a central checkpoint. The bcl-2 family consists of both cell death promoters and cell death preventers. Bax is one of pro-apoptotic proteins, whereas bcl-2 is one of anti-apoptotic proteins. The ratio of anti- to pro-apoptotic proteins constitutes a central checkpoint. The bcl-2 family consists of both cell death promoters and cell death preventers.
molecules such as bcl-2/bax determines the response to a death signal\cite{18-20}. Bcl-2 seems to prevent apoptosis induced by many stimuli, for example, irradiation, FasL\cite{21}, TRAIL\cite{22} and deprivation of growth factor, and it has been shown to suppress cytochrome c (cyt.c) efflux from mitochondria, inhibit calcium release from the endoplasmic reticulum\cite{23-25}. Bax, which normally resides in the cytosol, translocates to mitochondria when triggered by certain stimuli, and changes to its active configuration as a dimerized integral mitochondrial membrane protein\cite{26}. Translocated bax has been shown to induce cyt.c release followed by caspase activation. Several studies have reported that increment of the ratio of bcl-2/bax protein can prevent the progression of apoptosis in myocardium after I/R\cite{27,28}. In the present study, it was shown that apoptosis began to occur in rat intestinal villus subjected to 45 min of ischemia followed by 30 min of reperfusion. Although gene transcription and translation of bcl-2 were both inhibited after reperfusion, the expression of bax was apparently enhanced. It was suggested that the decreased ratio of bcl-2/bax by ischemia and reperfusion might play a pivotal role in induction of apoptosis. Our study also showed that the number of apoptotic cells in intestinal villus insulted by I/R was significantly decreased by aFGF administration. aFGF could inhibit bax gene expression and enhance bcl-2 gene expression in comparison with normal saline treatment. So the increment of the ratio of bcl-2/bax by administration of aFGF might be one of mechanisms attenuating I/R-induced apoptosis. These results are consistent with the result by Coopersmith et al\cite{29} that transgenic rat expressing bcl-2 could suppress apoptosis induced by I/R.

Our study also found that apoptosis occurred at 30 min postreperfusion followed by a return to baseline levels by 24 h, suggesting that induction of intestinal apoptosis by I/R and mucosal recovery were rapid processes. The reason for this interesting kinetics of induction of mucosal cell death and restoration was unclear. There were two possible explanations. First, it may be that there is a time-dependent increase in apoptosis-promoting factors, including Bax, during ischemia and early phases of reperfusion, which rapidly decreases with prolonged reperfusion. Second, corresponding to the decline in apoptosis-promoting factors, there may be simultaneous induction of inhibitors of apoptosis, e.g., Bcl-2, during the later phase of reperfusion. The exact mechanism needs to be studied in depth.

In conclusion, the present study provides the preliminary evidence that the protective effects of aFGF against I/R in rat intestinal villus might be partially due to its ability to inhibit I/R-induced apoptosis. Furthermore, our data strongly suggest that aFGF exerts its antiapoptotic effect via regulating gene expression of bcl-2, bax and the ratio of bcl-2/bax proteins. The precise mechanism for the inhibition of intestinal I/R injury and attenuation of apoptosis afforded by aFGF requires further investigation.

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