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 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 protein expression, but also for immunohistochemical analysis to detect P53 and P21WAF-1 expression.

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. 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.

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 antia apoptotic 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[11-14]. 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. As a multiple function growth factor, aFGF is involved in embryo development and tissue repair. Previous studies have shown that intravenous administration of exogenous aFGF could improve the physiological functions of intestine after I-R injury. 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 °C. Rats were allowed free access to water and food. The animals were anesthetized with 3% sodium pentobarbital at 6982 ISSN 1007-9327 CN 14-1219/ R World J Gastroenterol November 28, 2005 Volume 11 Number 44

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 °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 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 °C for 20 min. Then, sections were blocked with 5% normal swine serum in PBS for 30 min at 25 °C, followed by incubation with primary antibodies at a concentration of 5 μg/mL overnight at 4 °C. 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
intestine (jejunum). The time, when the animals were killed
sections prepared from the middle quarter of the small
reperfusion, TUNEL reaction was performed in serial
To quantify the extent of apoptosis after ischemia and
reperfusion. The structures of crypt and villus were both
aFGF on intestinal mucosa was very effective 2-12 h after
administration of aFGF. The protective function of
structure of the intestinal mucosa was markedly improved
During 6-12 h after reperfusion, the damage of intestinal
crypts just after the ischemic period. Two hours after
was small, with slightly edematous villus tips and intact
revealed that damage to the small intestine in I group
cyanotic color within 45 min. The histological evaluation
aorta, the entire small intestine showed a dark purple,
Histopathological findings
RESULTS
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
daughter, 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       | C group | R group |
|--------------|---------|---------|
| 2 h after reperfusion | 50.67±6.95 | 22.00±4.60 |
| 15 min after reperfusion | 29.50±5.61 | 25.17±6.43 |
| 30 min after reperfusion | 26.00±4.02 | 26.00±4.02 |
| 1 h after reperfusion | 34.67±5.47 | 29.83±7.08 |
| 6 h after reperfusion | 50.67±6.95 | 41.17±4.97 |
| 12 h after reperfusion | 54.17±4.86 | 42.83±2.32 |
| 24 h after reperfusion | 64.33±6.47 | 53.33±6.92 |
| 48 h after reperfusion | 28.50±5.47 | 23.33±3.83 |

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 P21WAF-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            | R group       | A group       | R group       | A group       |
|-------------------|---------------|---------------|---------------|---------------|
|                   | P53           | P21WAF-1      | P53           | P21WAF-1      |
| C group           | 23.67±4.55    | 23.67±4.55    | 32.50±3.94    | 32.50±3.94    |
| 1 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    | 32.50±3.27    | 39.83±4.58    | 37.83±4.36    |
| 2 h after reperfusion | 40.33±3.50    | 33.83±5.04    | 49.33±4.18    | 41.83±6.65    |
| 6 h after reperfusion | 50.50±4.23    | 42.67±3.88    | 53.00±4.29    | 44.67±4.66    |
| 12 h after reperfusion | 45.67±5.65    | 37.50±4.81    | 57.50±3.62    | 49.33±4.37    |
| 24 h after reperfusion | 30.00±6.13    | 25.33±3.33    | 34.50±4.39    | 30.00±3.79    |
| 48 h after reperfusion | 28.17±8.89    | 25.67±4.32    | 33.83±4.32    | 30.67±4.18    |

aP<0.05 vs C group; cP<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            | R group       | A group       | R group       | A group       |
|-------------------|---------------|---------------|---------------|---------------|
|                   | P53           | P21WAF-1      | P53           | P21WAF-1      |
| C group           | 23.5±6.0      | 22.4±2.5      | 26.7±2.0      | 24.6±4.1      |
| 1 group           | 38.9±5.1      | 34.8±4.2      | 32.6±2.0      | 31.7±2.0      |
| 15 min after reperfusion | 53.9±4.9      | 45.8±10.7     | 33.4±2.3      | 24.0±3.4      |
| 30 min after reperfusion | 34.0±4.4      | 41.9±5.2     | 23.3±4.2      | 22.9±2.0      |
| 1 h after reperfusion | 48.8±5.2      | 44.7±5.4      | 24.8±2.3      | 23.8±3.2      |
| 2 h after reperfusion | 46.7±5.4      | 41.3±6.3      | 26.5±2.7      | 24.9±3.0      |
| 6 h after reperfusion | 48.8±5.2      | 38.3±4.7      | 30.8±3.0      | 20.9±2.6      |
| 12 h after reperfusion | 51.0±4.5      | 34.1±4.8     | 32.7±1.6      | 21.9±2.8      |
| 24 h after reperfusion | 55.3±7.3      | 56.9±5.3      | 28.8±3.1      | 28.5±5.0      |
| 48 h after reperfusion | 56.4±8.3      | 61.0±4.0      | 29.0±2.3      | 28.9±3.8      |

aP<0.05 vs C group; cP<0.05 vs R group at matched time point.
demonstrated that I-R causes both mucosal and vascular to the deleterious effects of I-R, and it has been clearly recognized that the small intestine is extremely sensitive circulation are hemorrhage and ischemia. It is well

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\cite{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.

Expression characteristics of P53 and P21WAF-1 mRNA

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).

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

Expression of P53, P21WAF-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 1-6: 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.

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. 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. 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-1 gene 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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Science Editor Wang XL, Li WZ and Guo SY Language Editor Elsevier HK