Enhanced anti-apoptosis and gut epithelium protection function of acidic fibroblast growth factor after cancelling of its mitogenic activity

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Abstract

AIM: Mitogenic and non-mitogenic activities of fibroblast growth factor (FGF) are coupled to a range of biological functions, from cell proliferation and differentiation to the onset of many diseases. Recent reports have shown that acidic fibroblast growth factor (aFGF) has a powerful anti-apoptotic function, which may have potentially therapeutic effect on gut ischemia and reperfusion injuries. However, whether this function depends on its mitogenic or non-mitogenic activity remains unclear. In this study, we identified the source of its anti-apoptotic function with a mutant, aFGF28-154 and observed its effect on reducing gut ischemia and reperfusion injury.

METHODS: aFGF28-154 was generated by amplification of appropriate DNA fragments followed by subcloning the products into pET-3c vectors, then they were expressed in BL21 (DE3) cells and purified on an M2 agarose affinity column. This mutant aFGF28-154 maintained its non-mitogenic activity and lost its mitogenic activity. With a dexamethasone (DEX)-induced mouse thymocyte apoptosis model in vitro and in vivo, we studied the anti-apoptotic function of aFGF28-154. Also, in vivo study was performed to further confirm whether aFGF28-154 could significantly reduce apoptosis in gut epithelium after gut ischemia-reperfusion injury in rats. Based on these studies, the possible signal transduction pathways involved were studied.

RESULTS: With a dexamethasone (DEX)-induced mouse thymocyte apoptosis model in vitro and in vivo, we found that the anti-apoptotic function of aFGF28-154 was significantly enhanced when compared with the wild type aFGF. In vivo study further confirmed that aFGF28-154 significantly reduced apoptosis in gut epithelium after gut ischemia-reperfusion injury in rats. The mechanisms of anti-apoptosis function of aFGF28-154 did not depend on its mitogenic activity and were mainly associated with its non-mitogenic activities, including the intracellular calcium ion balance protection, ERK1/2 activation sustaining and cell cycle balance.

CONCLUSION: These findings emphasize the importance of non-mitogenic effects of aFGF, and have implications for its therapeutic use in preventing apoptosis and other injuries in tissues and internal organs triggered by ischemia-reperfusion injury.

INTRODUCTION

Fibroblast growth factors (FGFs) are members of a family of polypeptides that are potent regulators of cell proliferation, differentiation, and function. They play very important roles in normal development, maintenance of tissues, wound healing and repair. They have also been implicated in a wide range of pathological conditions, including tumorigenesis and metastasis[1-4]. Acidic and basic fibroblast growth factors (aFGF and bFGF), the important members of this family, were named for their different isoelectric points. They have similar molecular weights and spectra of biological activities, and show approximately 55% amino acid similarity. aFGF and bFGF stimulate the proliferation of all cells of mesodermal organ, and many cells of neuroectodermal, ectodermal, and endodermal origin. Also, both FGFs are chemotactic and mitogenic for endothelial cells in vitro, inducing production of many factors involved in regulation of many functions[2-3]. Recent reports have shown that aFGF has a powerful anti-apoptotic function, which may have potentially therapeutic use in some diseases[5-13]. However, whether this function depends on its mitogenic or non-mitogenic activity remains unclear, and clarification of this function may help to find its new mechanisms and active domains. In the present study, we modified the wild type of aFGF and acquired a mutant, aFGF28-154, which kept its non-mitogenic activity and lost its mitogenic activity. By using the dexamethasone (DEX)-induced apoptotic model, we found that the anti-apoptosis function of the modified aFGF28-154 was significantly enhanced when compared with the wild type of aFGF. The results indicated that the powerful anti-apoptosis function of aFGF28-154 was associated with the intracellular calcium ion balance protection and ERK1/2 activation sustaining, which did not depend on its mitogenic activity. Further studies showed that aFGF might have a surveillance function at the checkpoint of cell cycle, for determination of stagnation, division or apoptosis. Finally, in vivo study confirmed that aFGF28-154 could significantly reduce apoptosis in gut epithelium after gut ischemia-reperfusion injury in rats.

MATERIALS AND METHODS

Protein preparation

Plasmids encoding aFGF28-154 were generated by amplification of appropriate DNA fragments, followed by subcloning the
products into pET-3c vectors. aFGF28-154 protein was expressed in BL21 (DE3) cells and purified on an M2 agarose affinity column (Sigma). We carried out SDS-PAGE and Western blot analysis with aFGF monoclonal antibody (Sigma) to determine the accuracy of protein expression.

**Mitogenic activity assay**

MTT method was conducted to test the mitogenic activity of both aFGFs. NIH 3T3 fibroblasts and PC12 cell lines from Wuhan University were cultured in DMEM (Hyclone) with 150 mL/L fetal bovine serum (Gibco) plus 50 mL/L equine serum (Hyclone) for PC12 culture. These cells were stimulated with aFGF and aFGF28-154 at a final concentration of 0, 1, 2, 3, 4, 5, 6, 8, 10 ng/mL for 24 h before A value detection.

**Apoptosis models in thymocytes and treatment methods in vitro**

Twenty homogenous Balb/c mice aged four weeks were randomised into 4 groups: control group (group 1); DEX group (group 2), DEX+aFGF (Biopharmaceutical R&D Centre) group (group 3) and DEX+aFGF28-154 (group 4). DEX, if applied, was taken at a final concentration of 1 µg/kg, and DEX+aFGF10 µg/kg, and DEX+aFGF28-154 10 µg/kg. Animals were fed for 5 h before collection of thymocytes.

**Flow cytometry**

Flow cytometry was performed using an FACScaliber (Becton Dickinson). Phosphatidylinerse (PS), a sign of early apoptosis, was stained with Annexin V-FITC (Baosai), while DNA fragments were stained with PI apoptosis. Flow cytometry was performed using an FACScaliber (Becton Dickinson) to test the mitogenic activity of both aFGFs.

**Morphological observation and gel electrophoresis analysis of apoptosis**

Transmission electron microscope was used to morphologically visualize apoptosis and agarose gel electrophoresis was performed to find the damage of nuclear chromatin DNA.

**Analysis of intracellular Ca^{2+} concentration**

Confocal microscope was used to analyse the changes of intracellular Ca^{2+} concentration.

**Apoptosis model in gut epithelium and treatment methods in vivo**

Rat intestinal ischemia-reperfusion (I/R) injury was produced by clamping the superior mesenteric artery (SMA) for 45 min after reperfusion for 24 h. One hundred and fourteen Wistar rats were divided randomly into four groups, namely intestinal I/R plus saline treatment group (I/R+Saline), intestinal I/R plus wild type aFGF treatment group (I/R + aFGF), intestinal I/R plus aFGF 28-154 group (I/R plus aFGF28-154) and normal control group (normal control). In groups 1, 2 and 3, SMA was separated and occluded for 45 min, then released for reperfusion for 24 h. At the onset of reperfusion, saline (0.5 mL) or aFGF (4 µg/rat), or aFGF28-154 (4 µg/rat) was injected through the jugular vein. In control group, SMA was separated without occlusion. After the animals were sacrificed, tissue biopsies were taken from the intestine at 30 min, 1, 2, 6, 12, and 24 h, respectively. Apoptosis in epithelium entericum was assayed with TUNEL technique and analyzed using light microscope. All animal experiments were carried out according to the Guidelines for Care and Use of Experimental Animals and approved by the internal ethical committees of the Trauma Centre of Postgraduate Medical College (Beijing).

**Analysis of signal transduction in cells stimulated by DEX and both aFGFs in vitro**

Cultured thymocytes were sampled to carry out Western blot analysis. Antibodies used were ERK1/2, p-ERK1/2, JNK1/2, p38 MAPK, c-Myc (Sigma), and Caspase-3 (Boster).

**RESULTS**

**Protein preparation and mitogenic activation assay**

Plasmids encoding aFGF28-154 were generated by amplification of appropriate DNA fragments, followed by subcloning the products into pET-3c vectors. aFGF28-154 protein was expressed in BL21 (DE3) cells and purified on an M2 agarose affinity column (Sigma). We carried out SDS-PAGE and Western blot analysis with aFGF monoclonal antibody (Sigma) to determine the accuracy of protein expression (Figure 1A, B). We tested both aFGF and aFGF28-154 for their effects on proliferation of NIH 3T3 fibroblasts and rat pheochromocytoma (PC12) cells. The results indicated that the latter significantly lost its mitogenic activity by MTT results (Figure 1C). Thus, we could perform a comparative research to clarify whether aFGF function depended on mitogenic activity.
Assay of anti-apoptotic activation of both aFGFs

We examined the anti-apoptotic effects of both aFGFs by a DEX-induced thymocyte apoptosis model in mouse, which was considered as one of the best characterized apoptosis research systems[14-16]. First, a number of typical apoptotic thymocytes with apoptotic bodies from the DEX-induced group were visualized by transmission electron microscope (Figure 2A, B). Second, agarose gel electrophoresis results of this group also indicated characteristic ladder bands of oligonucleosomal fragments (180-200 bp) produced by extracted chromatin DNA, while few were found in blank group and the DNA damage in DEX -induced group was much more serious than that in both aFGFs treated groups (Figure 2C). Third, Annexin V-FITC/PI staining was used to distinguish apoptotic and necrotic cells from normal ones by flow cytometry (Figure 2D). Our test indicated that under the stimulation of DEX, thymocytes showed apoptosis both in vivo and in vitro (44.40% and 20.07%, respectively) after 5-h culture. With the protection of aFGF, when exposed to DEX, the apoptosis rate was lower both in vivo and in vitro (33.71% and 18.31%, respectively). Under the same condition, however, the apoptosis rate was significantly lower in aFGF28-154 group than in DEX and aFGF groups both in vivo and in vitro (23.95% and 13.80%, respectively) (Figure 2D).

The quantitative analysis of anti-apoptosis results and the dose-effect relationship of aFGF28-154 are summarized in Table 1. Fourth, we studied the changes of concentration of intracellular calcium ion (Ca2+) by confocal microscope to further confirm the anti-apoptotic effects of both aFGFs on mouse thymocytes. High concentration of intracellular Ca2+ caused by influx increase was involved in triggering protease and endonuclease

![Figure 2](image-url)
activation and apoptosis. Hence, Ca$^{2+}$ influx was considered as a sign of apoptosis occurrence[17-19]. In this study, the intracellular Ca$^{2+}$ concentration in thymocytes increased quickly when exposed to DEX. When administrated with aFGF, however, Ca$^{2+}$ concentration decreased to a lower level (Figure 3A). However, the test for aFGF28-154 showed a marked reduction of Ca$^{2+}$ influx (Figure 3B).

**Table 1** In vitro quantitative analysis of anti-apoptotic effects and dose-effect relationship of aFGF28-154 on thymocytes induced by DEX (mean±SD)

| Groups          | Conc. (ng/mL) | Apoptosis (%) |
|-----------------|---------------|---------------|
| Normal control  |               | 1.37±0.39     |
| DEX only        |               | 18.99±0.52    |
| DEX plus aFGF28-154 | 50          | 16.14±0.44    |
|                 | 100           | 12.35±0.36    |
|                 | 150           | 9.75±0.20     |
|                 | 200           | 11.23±0.71    |

Table 2 In vivo quantitative analysis of apoptosis in epithelium entericum in different groups (mean±SD)

| Time               | Normal control (n = 5) | I/R+Saline (n = 36) | I/R+aFGF (n = 36) | I/R+aFGF 28-154 (n = 36) |
|--------------------|------------------------|---------------------|-------------------|--------------------------|
| Before ischemia    | 5.83±1.47              | 5.83±1.47           | 5.83±1.47         | 5.83±1.47                |
| I/R 0 min         | 29.83±3.43             | 23.83±2.78          | 25.50±1.87        | 25.50±1.87               |
| I/R 60 min        | 39.33±4.32             | 30.33±3.07          | 33.44±5.60        | 33.44±5.60               |
| I/R 2 h           | 46.16±4.06             | 39.66±3.56          | 40.66±2.73        | 40.66±2.73               |
| I/R 6 h           | 31.33±3.72             | 25.5±2.16           | 26.00±2.61        | 26.00±2.61               |
| I/R 12 h          | 62.5±3.08              | 48.50±2.74          | 50.16±3.71        | 50.16±3.71               |
| I/R24 h           | 51.66±3.87             | 41.33±2.94          | 42.16±3.31        | 42.16±3.31               |

$^aP<0.05$ vs normal control; $^bP<0.05$; $^cP<0.01$ vs DEX only.

**Gut epithelium apoptosis and treatment effects with both aFGFs in vivo**

We investigated the therapeutic use of both aFGFs in gut ischemia-reperfusion injury in rats and found that the apoptosis rate of epithelium entericum was 5.83±1.47 in normal control rats, 46.16±4.06 in rats treated with saline, 39.66±3.56 in rats treated with wild type of aFGF and 40.66±2.73 in rats treated with aFGF28-154 2 h after ischemia-reperfusion injury (Table 2). The quantitative analysis of apoptosis in epithelium entericum in different groups of different time points is summarized in Table 2. The morphological examination with light microscope and immunohistochemical detection and quantification of apoptosis based on the labelling of DNA strand breaks confirmed that tissue edema, local necrosis (Figure 4A, C, E, G) and apoptosis in epithelium entericum were markedly reduced in both aFGFs treated rats (Figure 4B, D, F, H).

**Analysis of possible signal transduction pathway**

In in vitro study, we analysed the changes of ERK1/2, p-ERK1/2, JNK1/2, p38 MAPK, c-Myc and Caspase-3. The results showed that only ERK1/2 pathway was activated in all of the three pathways during aFGF anti-apoptosis course (Figure 5A, B, C, D) and no phosphorylated JNK1/2 and p38 MAPK could be detected. In normal control thymocytes, steady ERK1/2 activation was observed, and such an activation was almost completely blocked when cells exposed to DEX. But both aFGFs attenuated this DEX-induced blockage, maintaining ERK1/2 activation, which is required for normal thymocyte cycle at a certain level (Figure 5A). P38 expression in control group was steady with time lapse (Figure 5B) and decreased gradually when cells exposed to DEX (Figure 5C). However, the total p38 MAPK expression decreased at 30 min and then a peak expression appeared at 60 min when the DEX-induced cells were protected with aFGF or aFGF28-154 (Figure 5D, E). The expression of c-Myc and Caspase-3 in thymocytes in the control group was kept at a stable level following the time course (Figure 5B). When stimulated merely by DEX, c-Myc was highly expressed in the first 30 min and the activated Caspase-3 increased following c-Myc (Figure 5C). These results indicated that DEX-induced thymocyte cell cycle turned out to be c-Myc-induced apoptosis. In the aFGF group, however, c-Myc was expressed only at 0 min, and then showed no significant change at other time points (Figure 5D). In the aFGF28-154 group, c-Myc showed a peak expression at 30 min, and then held at a relatively steady level (Figure 5E).

**DISCUSSION**

Currently, the anti-apoptotic effects of aFGFs have been established from the results of DNA ladder electrophoresis, Annexin V-FITC/PI staining, examination of Ca$^{2+}$ concentration with confocal microscope, and in vivo study on gut ischemia-reperfusion injury in rats. But the results were far beyond our expectation. aFGF, especially the modified non-mitogenic aFGF28-154 with a powerful ability to reduce the apoptosis rate, made use to explore whether there were some special mechanisms involved in such an action.

To further investigate the mechanisms of the anti-apoptotic function of aFGF, we studied three protein kinases, including...
extracellular-signal-regulated kinase (ERK) 1/2, c-Jun-NH2-terminal kinase (JNK) 1/2, and p38 mitogen associated protein kinase (MAPK). According to the Western blot analysis on phosphorylation, we found that only ERK1/2 pathway was activated in all of the three pathways during aFGF anti-apoptosis course (Figure 5A) and no phosphorylated JNK1/2 and p38

Figure 4 Morphological examination and immunohistochemical detection and quantification of apoptosis based on the labelling of DNA strand breaks (Roche Applied Science, Germany). A: Marked epithelial separation from the basement, subepithelial edema, haemorrhage, erosion. C: Necrosis in I/R plus saline control group. E and G: Tissue damage reduction in both aFGF and aFGF28-154 treated groups. B: Immunohistochemical detection and quantification of apoptosis in normal epithelium entericum. D: Increased number of apoptotic cells in epithelium entericum in rats treated with saline. F and H: Significant reduction of apoptotic cells in epithelium entericum in rats treated with both aFGF and aFGF28-154.

Figure 5 Function of aFGFs as a checker of cell cycle. A: Western blot analysis of the phosphorylation of ERK1/2. B: Western blot analysis of ERK1/2 MAPK, JNK1/2 and p38 MAPK, as well as c-Myc and Caspase-3 in control group. C: Analysis of thymocytes stimulated by DEX. D: Analysis of thymocytes under aFGF protection. E: Analysis of thymocytes under aFGF28-154 protection.
MAPK could be detected. In normal control thymocytes, the steady ERK1/2 activation was observed, and such an activation was almost completely blocked when cells exposed to DEX. But both aFGFs attenuated this DEX-induced blockage, maintaining ERK1/2 activation, which is required for normal thymocyte cycle at a certain level (Figure 5A). ERK1/2 activation was crucial for aFGF to exert protective effects on many cell types[20,21]. DEX could induce mitochondrial membrane potential collapse and promote Ca2+ release from intracellular pool and the subsequent stimulation of a Ca2+ influx from extracellular environment, which contributes to apoptosis[16]. However, an ERK1/2 activation could protect mitochondrial membrane potential by downstreaming Bcl-X activation[22]. The fact that only ERK1/2 pathway was activated in all of the three pathways and Ca2+ influx reduction in both aFGF groups strongly suggested that the anti-apoptotic activity of aFGF involved in maintaining ERK1/2 activation and regulation of intracellular Ca2+ concentration.

A very interesting finding from the results of total p38 MAPK expression attracted our great attention. p38 expression in control group was steady with time lapse (Figure 5B) and decreased gradually when cells exposed to DEX (Figure 5C). However, the total p38 MAPK expression decreased at 30 min and then a peak expression appeared at 60 min when the DEX-induced cells were protected with aFGF or aFGF28-154 (Figures 5D, E). Normally, the activation of p38 MAPK often follows stress induction[23]. Although p38 MAPK was not markedly activated in all groups in our study, we noticed that according to a previous study by Lasa et al, dexamethasone could cause sustained expression of MAPK phosphatase 1 and phosphatase-mediated inhibition of p38 MAPK[24]. We also found p38 MAPK was constantly decomposed by DEX induction (Figure 5C). Because the cells in control group were not stressed and the other 3 groups were all exposed to DEX, we speculated that the peak expression of p38 MAPK at 60 min in both aFGF groups might reflect an unveiled stress, which was lagged in both aFGF groups compared with the DEX group.

To investigate the anti-apoptotic function of aFGFs at protein level, we further examined the changes of c-Myc and Caspase-3. Thymocytes in control group were kept at a stable c-Myc level following the time course (Figure 5B). When stimulated merely by DEX, c-Myc was highly expressed in the first 30 min and the activated Caspase-3 increased following c-Myc (Figure 5C). These results indicated that DEX-induced thymocyte cell cycle turned out to be c-Myc-induced apoptosis. In aFGF group, however, c-Myc was expressed only at 0 min, and then showed no significant change at other time points (Figure 5D). In aFGF28-154 group, c-Myc showed a peak expression at 30 min, and then held at a relatively steady level (Figure 5E). c-Myc, in association with its partner Max, could function as a transcription factor to drive apoptosis when low quantities of survival factors such as IGF-1 and aFGF were present[25,26]. These results indicated that cell cycle was stagnated in aFGF group, and balanced in aFGF28-154 group. In both aFGF groups, Caspase-3 activation remained nearly null during first 30-60 min and was suddenly enhanced at 60 min and still kept at a high level until 180 min (Figure 5D, E). This activation is cohort with the p38 reaction during stress stimulation and especially with c-Myc expression in normal and both aFGF groups.

The above signal transduction analysis suggested that aFGF might serve as a “checker” for cell cycle while exerting anti-apoptotic function. These functions, including stagnation determination, cell division or apoptosis induction may be similar to those of p53, a typical checker of G1, S and G2/M phases of cell cycle, which contribute both to the cell cycle arrest and apoptotic functions by preventing replication of cells from DNA damage[27-30]. Our results illustrated that when exposed to apoptosis inducers, aFGF would also induce cell cycle arrest.

This action was reflected by the stagnation of c-Myc expression in our study (Figures 5D, E). At the time interval of null c-Myc expression, thorough check up and repair might be conducted, thereby, new c-Myc would be expressed by aFGF induction. We regarded that this procedure as a “sentence procedure”, reflected by p38 implied second stress, might be carried out by the combined effects of c-Myc and Caspase-3. C-Myc is a typical two-face protein, which is necessary not only for DNA-cleavage in G2 phase of cell cycle, but also for cell proliferation, when cells are protected by trophy factors[31]. If cells can pass through the examination and undergo a thorough “overhaul”, they would be kept in normal cell cycle; whereas other non-recoverable cells would be “sentenced to death” with high activation of intracellular procaspase-3 by newly expressed c-Myc. Caspase-3 may finally cleave the damaged DNA and execute apoptosis.

Here we conclude that the anti-apoptotic function of aFGFs was enhanced after cancelling of its mitogenic activity and the anti-apoptotic function did not depend on its mitogenic activity. The action is associated with the protection of intracellular Ca2+ concentration balance, ERK1/2 pathway sustaining, and cell cycle balance. During the process of anti-apoptosis, aFGF may play a role as a “checker” for cell cycle. Furthermore, we speculate that there are two possible reasons for the anti-apoptotic function enhancement after aFGF modification. First, aFGF28-154 loses the ability of nuclear translocation, a typical aFGF characteristic[32], causing a longer interaction period with FGF receptors and higher efficiency for aFGF28-154 than the wild one. Second, the structural change of aFGF28-154 may be better exposed with its active domain, which contributes to some anti-apoptosis related activities.

aFGF has been widely used in the fields of wound repair and regeneration and some other fields[32,33]. Because of its extensive distribution and broad mitogenic functions, the side-effects or potential tumour induction is of great concern for doctors. In this study, we demonstrate a genetically engineered method to abolish the mitogenic activity and maintain the non-mitogenic activity of aFGF. This may be helpful to reduce people’s worry about its tumour induction and to take as many preventive measures as possible clinically. In short, the powerful anti-apoptotic function of aFGF28-154 may play an important role in prophylaxis and treatment of some immune system diseases or as a protector in preventing apoptosis in tissues and organs triggered by ischemia and reperfusion injury.

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