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Original Research Article

Protective Effect of Modified Human Acidic Fibroblast Growth Factor against Actinomycin D-Induced NRK52E Cells Apoptotic Death

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Abstract

Purpose: *To investigate whether modified acidic fibroblast growth factor (MaFGF) can protect NRK52E cell against apoptotic death induced by actinomycin D (Act D) and the effect of MaFGF on PI3K/Akt signaling pathway.*
Methods: *NRK52E cell apoptotic death was measured by several methods including cell morphologic observation, Hoechst 33342 staining and flow cytometry. In addition, the levels of phosphorylated-Akt protein were analyzed by Western blotting method.*
Results: *The results showed that 0.75 mg/L Act D-treated NRK52E cell for 20 h was the optimal conditions for establishing NRK52E cell apoptotic model. Different doses of MaFGF (0.01, 0.03, 0.1, 0.3 and 1.0 mg/L) decreased apoptotic rate but enhanced the expression of phosphorylated Akt protein. However, MaFGF's protection against Act D-induced apoptosis was significantly ($p < 0.05$) prevented when NRK52E cells were exposed to wortmannin.*
Conclusion: *These results reveal that MaFGF can reduce the level of ActD-induced apoptotic cell death in 20 h, and the protective mechanism of MaFGF may be associated with the activation of PI3K/Akt signaling pathway by up-regulation of expression of phosphorylated Akt protein.*

Keywords: *Modified acidic fibroblast growth factor (MaFGF), Renal injury, Apoptotic death, Actinomycin D (Act D)*

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INTRODUCTION

Actinomycin D (Act D), as a transcriptional inhibitor, can inhibit protein synthesis. Recent studies have shown that Act D, at a concentration of 0.8 - 1.0 μg/ml or higher, causes typical apoptotic cell death in a variety of cell lines [1,2]. Consequently, Act D has been used for establishing cell apoptotic models.

It has been reported that the mechanism of Act D-induced apoptotic death is closely related to PI3K/Akt signaling pathway, which includes PI-3K, PKB/Akt and 70s6K [3]. The activation of Akt, known as protein kinase B (PKB), is an important prerequisite in PI3K/Akt signaling pathway, which involves a series of activities such as regulating cell apoptosis, proliferation, differentiation and metabolism. Andreucci *et al* suggested that PI3-kinase/Akt signaling pathway plays an important role in the regulation of renal repair after ischemia/reperfusion injury [4]. Xie *et al* demonstrated that wortmannin, which is the special inhibitor of PI3K, may increase the levels

of serum creatinine and urea nitrogen, and enhance the degree of renal injury during kidney ischemia-reperfusion injury in mice; they also suggested that there was a relationship between PI3-kinase/Akt signaling and renal injury [5].

Acidic fibroblast growth factor (aFGF) is one of the members of the FGFs family with many biological effects, such as promotion of cell proliferation, growth and differentiation. It has been proved that aFGF exhibits mitogenic and non-mitogenic activities, and its powerful mitogenic activities have been implicated in some pathological processes including hyperblastosis, tumorigenesis and metastasis [6,7]. It has previously been reported that an N-terminal nuclear localization sequence (NLS) in aFGF is required for full mitogenic activity. Modification of the N-terminal of aFGF gene and elimination of N terminal residues 1–27 plus methionine substitution of leucine at the site of 27 yields a human acidic fibroblast growth factor mutant (haFGF27-154), that is, modified aFGF (MaFGF) [8,9]. It has been shows that when the N-side 1 to 27 amino-acid residue of aFGF is removed, its mitogenic activity is lost or obviously decrease, but its non-mitogenic effect still exists [9-12].

Our previous studies have demonstrated that MaFGF can prevent renal ischemia-reperfusion injury in rats [13]. Whether MaFGF has protective effect on renal tubular epithelial cells has not been reported. The present study was undertaken assess the effect of MaFGF on a normal rat kidney proximal tubule epithelial cell line (NRK52E) cell apoptosis induced by Act D, and also to study the effect of MaFGF on PI3K/Akt signaling pathway.

EXPERIMENTAL

Reagents

MaFGF was supplied by Biopharmaceutical Research and Development Center of Jinan University, China. Before use, it was diluted with high-glucose (4.5 g/L glucose) Dulbecco's modified Eagles Medium (DMEM, Gibco, USA) to the required concentration. Act D and wortmannin were purchased from Sigma Chemical Co (St Louis, MO, USA). Specific antibodies for total Akt or phosphorylated Akt (Ser473) were purchased from Cell Signaling (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively, while and Hoechst 33342 dye was purchased from Sigma Chemical Co (St Louis, MO, USA).

Cell line

NRK52E cell line, a normal rat kidney proximal tubule epithelium line, from the kidney disease Institution of Sun Yat-Sen Medical University, were cultivated *at 37 °C* in an atmosphere *containing 5 % CO2*, *95 % air* and *relative humidity* of *98%*. The culture medium high glucose (DMEM, Gibco, USA) contained 100 g/L fetal bovine serum (heat inactivated), 10 U/ml penicillin and 100 mg/ml streptomycin, and the cells were transferred to fresh culture medium every 2 - 3 days.

Cell apoptosis assay

Samples were prepared for flow cytometry as described previously.[14] Briefly, treated cells (5×10^5/well) were digested by 0.25 % trypsin, harvested and rinsed with PBS 3 times, fixed with 70 % ethanol at 4 ^{0}C overnight. The fixed cells were rinsed with PBS 3 times, treated with RNAse at 37 ^{0}C for 30 min, and then stained by propidium iodide (PI) at 4 ^{0}C for 30 min. Finally, apoptosis rates were detected with flow cytometry (FCA-SCalibur, Becton Dickinson, USA).

Hoechst 33342 Staining

Samples were prepared for Hoechst 33342 staining as described previously [14]. Briefly, coverslips with adherent treated NRK52E cell were collected at specified time points and washed, and the cells were fixed with 4 % paraformaldehyde at 4 ^{0}C for 10 min and stained with Hoechst 33342 (5 µg/ml) for 20 min at room temperature in the dark. They were rinsed with PBS for 3 times, the cells were viewed with a fluorescence microscope equipped with a UV filter (Nikon, Tokyo, Japan). The images were recorded on a computer with a camera attached to the microscope. The Hoechst reagent was taken up by the nuclei of the cells and apoptotic cells exhibited a bright blue fluorescence.

Western blot analysis

Akt expression and phosphorylation were examined by Western blotting analysis according to previously published methods [15]. Briefly, protein 40 µg was spotted per lane and subjected to 12% SDS-PAGE and subsequently transferred to PVDF membrane. The membranes were blocked with 5% skimmed milk and sequentially incubated with phosphorylated Akt antibody or anti-Akt overnight at 4°C, followed by incubation with IgG-HRP conjugated secondary antibody (1:1000 dilution) at room temperature for 1.5 hrs and rinsed with TBST (TBS containing

0.1 % Tween 20, 3 × 10 min). The blots were detected by enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia) s and the images were scanned by gel image analysis system. β-actin was used as a reference for making quantitative comparison.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using the SPSS software package (version 10.0, SPSS Company, USA). Differences between groups were assessed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significance.

RESULTS

Actinomycin D-induced NRK52E cell apoptosis

The morphological change in Act D-induced NRK52E cell apoptosis is shown in Figure 1. In the control group, the cells looked like a cobblestone – irregular appearance and compact arrangement. For the 0.5 mg/L and 0.75 mg/L Act D groups, abnormal morphologic change of NRK52E cell observed. Some cellular nuclei condensed and broke into pieces and a few apoptotic bodies could be seen. Comparing the cell apoptotic results among all Act D-treated groups, 0.75 mg/L Act D treated for 20 h was a better process condition for inducing NRK52E cell apoptosis.

The cell apoptotic rate detected by flow cytometry indicated that NPK52E cells apoptosis induced by Act D at concentration of 0.25 mg/L, 0.5 mg/L, 0.75 was dose-dependent, and the concentration of 0.75 mg/L Act D was the optimal condition for inducing NPK52E cells apoptosis (Figure not shown).

Protective effect of MaFGF against Act D-induced NRK52E cell apoptosis

The results showed that pre-treatment with MaFGF at doses of 0.01, 0.03, 0.1, 0.3, 1.0 mg/L significantly protected NRK52E cell against Act D-induced apoptosis, as shown in Figure 2.

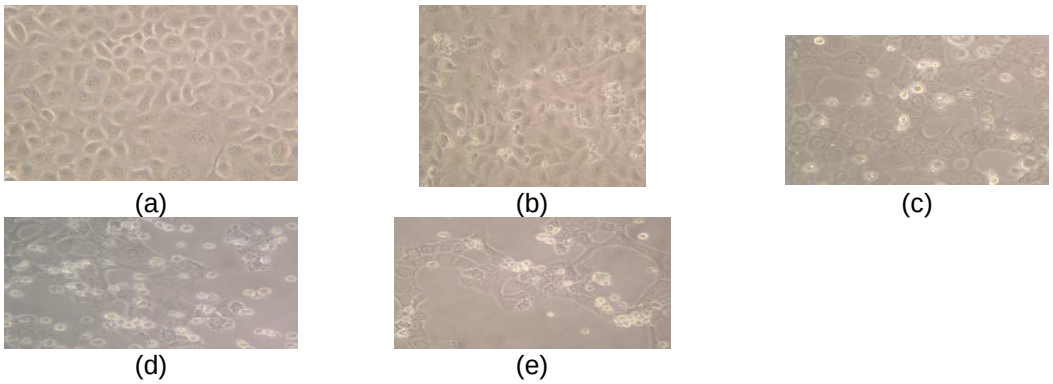

Fig 1: Morphological changes in NRK52E cells exposed to Act D for 20 h (×200). (a) Normal NPK52E cells. (b) NRK52E cells exposed to 0.25 mg /L Act D. (c) NRK52E cells exposed to 0.5 mg /L Act D. (d) NRK52E cells exposed to 0.75 mg /L Act D. (e) NRK52E cells exposed to 1.0 mg /L Act D.

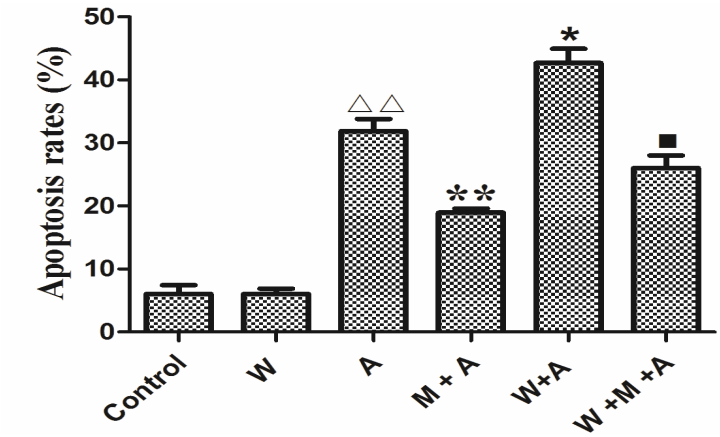

Figure 2: Protective effect of MaFGF on apoptosis of NRK52E cells exposed to 0.75 mg/L Act D for 20 h. Data are presented as mean ± SD (n = 5); $^{*}p < 0.01$ versus control group (no Act D); $^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$ versus model group (Act D alone)

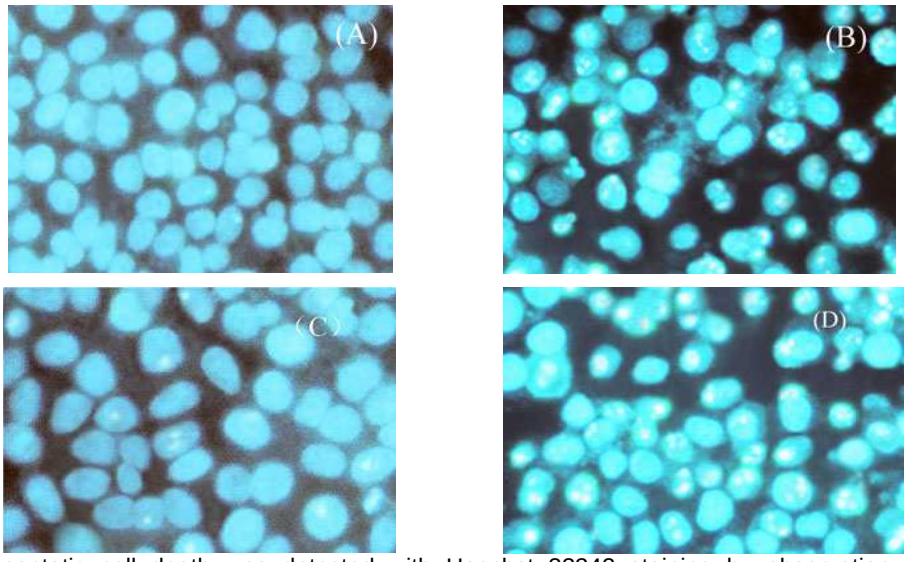

Figure 3: Apoptotic cell death was detected with Hoechst 33342 staining by observation of the nuclear condensation (×200). (A) normal cells; (B) cells treated with 0.75 mg /L Act D for 20 h; (C) cells pretreated with 0.1 mg/L MaFGF for 24 h and then treated with 0.75 mg /L Act D for 2 h; (D) cells pretreated with 200 nmol/L wortmannin for 2 h and then with 0.75 mg /L Act D for 20 h

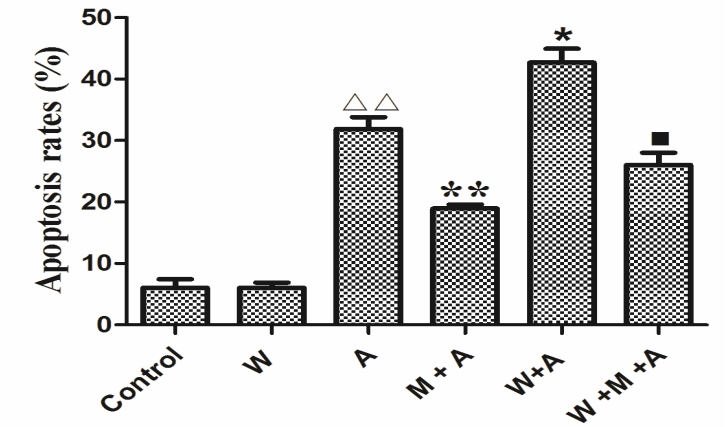

Figure 4: Influence of wortmannin on Act D-induced NRK52E cell apoptosis and on protection of MaFGF against Act D-induced NRK52E cell apoptosis. The apoptosis was measured by flow cytometry with PI staining. (W: Wortmannin; A: Act D; M: MaFGF). Data are presented as mean ± SD (n = 5). $^{\triangle\triangle}p < 0.01$ versus control group, $^{*}p < 0.05$, $^{**}p < 0.01$ versus A group, $^{\blacksquare}p < 0.05$ versus "W+A" group

The anti-apoptostc effect of MaFGF at 0.1 mg/L on Act D-indued apoptisis was also confirmed by Hoechst 33342 staining (Figure 4C), showing a significant decrease ($p < 0.05$) of the number of cells with nuclear condensation in MaFGF combined with Act D groups, when compared to that in Act D only group (Figure 3B).

Protective effect of MaFGF against Act D-induced apoptosis is associated with activtion of PI-3K/Akt signaling pathway

The results showed wortmannin did not increase cell apoptosis in control cells, but significantly (p <0.05), enhanced Act D-induced apoptosis, measured by flow cytometry (Figure 4). The enhanced effect of wortmannin on Act D-induced apoptosis was further confirmed by Hoechst 33342 staining (Figure 3D), which showed that the number of nuclear condensed cells in Act D combined with wortmannin group increased, compared to the group of Act D alone (Figure 3B). Pre-treatment with wortmannin could significantly block the protective effect of MaFGF against Act D-induced apoptosis (Figure 4).

The results of Western-blotting analysis showed that of total Akt expression was not changed in various groups (data not shown) and phosphorylated Akt expression was significantly (p <0.05) increased in MaFGF-treated cells (Figure 5). The relative phosphorylated Akt quantification related to β actin shown that expressions of phosphorylated Akt in MaFGF groups at doses of 0.1, 0.3 and 1.0 mg/L significant increased compared to the model

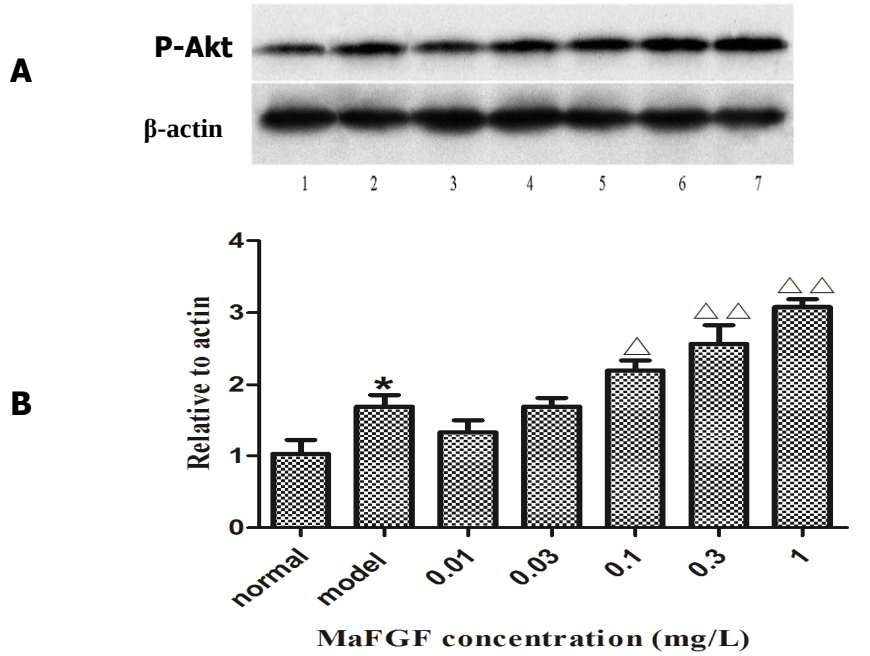

Fig 5: Effect of MaFGF on expression of phosphorylated Akt protein. ***Key***: (for A and B): 1 = control group (control group); 2 = model group (0.75 mg/L Act D); 3 - 7 = MaFGF (0.01, 0.03, 0.1, 0.3 and 1.0 mg/L, respectively); β-actin was used as a reference for making quantitative comparison (A). The data is presented as mean ± SD (n = 5). $^{\triangle}P<0.05$, $^{\triangle\triangle}<0.01$ versus model group; $^{*}P<0.05$ versus control group (B)

group. Thus, the effect of MaFGF on activated PI-3K/Akt signaling was related to up-regulation of phosphorylated Akt expression (Figure 5).

DISCUSSION

Renal tubular epithelial cell apoptosis has been suggested as the cause of kidney damage during renal ischemia-reperfusion, acute renal failure, drug poisoning and so on [16-18]. Therefore, protecting renal against injury and promoting renal repair after injury is a key treatment strategy in acute renal failure.

MaFGF is a mutant of human acidic fibroblast growth factor, which also is called non-mitogenic human acidic fibroblast growth factor because its N-terminal is modified and its mitogenic activity is obviously lower than that of aFGF [9,11]. Recent findings have shown that MaFGF has a variety of anti-apoptosis effects, such as antagonizing ischemia/reperfusion-induced intestinal epithelial cell apoptosis, inhibiting CCl_4-induced hepatocyte apoptosis, preventing H_2O_2-induced myocardial apoptosis, as well as resisting dexamethasone-induced thymocytes apoptosis [10,12,19,20]. However, whether MaFGF can protect NRK52E cell against apoptosis remains poorly understood.

In present study, we used Act D to induce NRK52E cell apoptosis and to investigate whether MaFGF can protect NRK52E cell against Act D-induced apoptosis. Treatment of NRK52E cells with different doses and time showed that 0.75 mg/L Act D for 20 h was the optimal condition for establishing a apoptosis model with characteristics such as stability, dose-reliability and ease of replication. The results demonstrate that MaFGF can protect NRK52E cell against Act D-induced NRK52E cell apoptosis in a dose-dependent manner.

Previous studies have revealed that PI-3K/Akt signaling pathway plays a very important regulatory role in renal ischemia-reperfusion injury. Akt, a crucial cell survival signal transducer, can inhibit apoptosis through phosphorylation and inactivation of the proapoptotic proteins [1,21]. It has already been found that pre-treatment with PI-3K inhibitors wortmannin increases the degree of injury of renal tubule, indicating that there is a relationship between renal injury and PI-3K/Akt signaling pathway [22-24].

We also investigated the relationship between the effect of MaFGF on NRK52E cell apoptosis and activation of PI3K/Akt signaling. The results demonstrate that the anti-apoptotic effect of MaFGF is associated with the activation of PI-3K/Akt signaling pathway. This conclusion was based on the following: (1) when the cells were pre-treated with wortmannin, the anti-apoptotic effect of MaFGF decreased; (2) treatment of

NRK52E cell with MaFGF for 24 h, Akt was hyperphosphorylated unlike the model group. These findings confirm that the protective effect of MaFGF against ActD was related to activation of PI-3K/Akt signaling because the anti-apoptosis ability of MaFGF was antagonized by wortmannin through inhibition of PI3K/Akt signaling, causing the cells to be more vulnerable to Act D damage. The increasing levels of phosphorylated Akt induced by MaFGF are consistent with the protective efficacy of MaFGF and thus further supports this conclusion.

Remarkably, our results show that the level of phosphorylated Akt protein increased in Act D model unlike in the normal group, suggesting that PI-3K/Akt signaling was activated when NRK52E cells were injured by Act D, which acted as an initiator of injury; this may be as a result of the body's own anti-injury regulation. This finding is similar to that reported earlier where it was found that phosphorylated Akt (activated form) level increased after renal ischemia-reperfusion injury in mice [5]. The higher levels of phosphorylated Akt protein in MaFGF groups demonstrate that MaFGF further facilitates the activation of PI3K/Akt signaling pathway.

CONCLUSION

MaFGF can protect NRK52E cell against Act D-induced apoptosis. This finding is consistent with our previous reports that MaFGF can antagonize H_2O_2-induced NRK52E cell apoptosis [25]. Increase in phosphorylated Akt after treatment with MaFGF is linked to anti-apoptosis of MaFGF. Therefore, activation of PI3K/Akt signaling plays a key role in the protection of MaFGF on NRK52E cell. Associated with the anti-apoptotic effect of MaFGF on other cells as mentioned above, it can be inferred that the anti-apoptotic effect of MaFGF on different cells have something in common. One of the anti-apoptotic mechanisms might be attributable to the activation of PI3K/Akt signaling pathway via enhancement of hyperphosphorylation of Akt protein.

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