**Protective effects of non-mitogenic human acidic fibroblast growth factor on hydrogen peroxide-induced damage to cardiomyocytes in vitro**

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Received: 2004-09-18 Accepted: 2004-12-26

**Abstract**

**AIM:** To study the protective effect of non-mitogenic human acidic fibroblast growth factor (FGF) on cardiac oxidative injury in vivo.

**METHODS:** Ventricular cardiomyocytes were isolated from 1- to 3-d-old neonatal SD mice and cultured in Dulbecco’s minimum essential medium supplemented with 15% fetal bovine serum under an atmosphere of 5% CO₂-95% air at 37 °C, as well as assessed by immunocytochemical assay. We constructed the cardiomyocyte injury model by exposure to a certain concentration of H₂O₂. Cellular viability, superoxide dismutase (SOD) activity, leakage of maleic dialdehyde and anti-apoptosis effect were included to evaluate the cardiac protective effect of non-mitogenic human acidic FGF.

**RESULTS:** Over 50% of the cardiomyocytes beat spontaneously on the 2nd day of culture and synchronously beat after being cultured for 3 d. Forty-eight hours after plating was completed, the purity of such cultures was 95% myocytes, assessed by an immunocytochemical assay. Cellular viability dramatically decreased with the increasing of the concentration of H₂O₂. Non-mitogenic human acidic FGF showed significant resistance to the toxic effect of H₂O₂, significantly increased the cellular viability as well as the activity of SOD, and dramatically decreased the leakage of maleic dialdehyde as well as the cellular apoptosis rate.

**CONCLUSION:** Hydrogen peroxide shows strong cytotoxicity to the cultured cardiac myocytes, and non-mitogenic human acidic FGF shows strong cardio-protective effect when exposed to a certain concentration of H₂O₂.

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**Key words:** Non-mitogenic human acidic fibroblast growth factor; Cardioprotection; Cardiomyocytes; H₂O₂

Lin ZF, Li XK, Lin Y, Wu F, Liang LM, Fu XB. Protective effects of non-mitogenic human acidic fibroblast growth factor on hydrogen peroxide-induced damage to cardiomyocytes in vitro. *World J Gastroenterol* 2005; 11(35): 5492-5497
http://www.wjgnet.com/1007-9327/11/5492.asp

**INTRODUCTION**

Fibroblast growth factor (FGF) is a family of at least 21 structurally and functionally relevant polypeptides characterized by a high affinity of heparin[1]. Acidic FGF found mainly in the brain and retina as well as in the cardiac tissue is also a multifunctional protein, which has many effects including mitogenesis and non-mitogenesis effects.

Studies indicate that intracoronary, intrapericardial, or myocardial administration of FGFs in chronically ischemic canine and porcine hearts can stimulate angiogenesis, a process to minimize infarct size and improve cardiac function[2]. At the same time, though there are some promising results in animal model trials, it produces acute negative inotropic effects on adult cardiac myocytes resulting from alterations in intracellular Ca²⁺ homeostasis[3]. Many people wonder if there would be a potential danger of stimulating the growth of tumor, since NIH-3T3 fibroblasts transfected to express constitutively a secreted form of FGF-1 that becomes tumorigenic[4,5]. FGF-1 behaves as a tumorogenic factor in the NBT-II bladder carcinoma cell model[6].

According to the function and structure of human aFGF, by the method of gene engineering, we removed the part of gene related to mitogenesis, eliminated or reduced the power of mitogenesis, and found it was expressed in...
Escherichia coli. We isolated the non-mitogenic human fibroblast growth factor (nm-haFGF) protein, and justified them by mAb of human FGF. Our relevant research results showed that, after modifying the structure of gene and protein, modified haFGF reduced its mitogenic effect, and preserved its other bio-effects including anti-apoptosis effect, cardio-protective effect, etc.\(^7\). In this paper, we isolated and cultured the primary cardiomyocytes from 1- to 3-d neonatal SD mice, and observed the pharmacological cardio-protective effect in vivo.

**MATERIALS AND METHODS**

**Drugs and reagents**

Nm-haFGF was recombined in our experiment laboratory. DMEM, fetal bovine serum (FBS) and trypsin were purchased from Hyclone Co. (UT, USA). Cell death detection kit and immunocytochemical assay kit were purchased from Boster Co. (Wuhan, China). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (St. Louis, MO, USA). Superoxide dismutase (SOD) and maleic dialdehyde (MDA) were purchased from Sigma (St. Louis, MO, USA). Super oxide dismutase (SOD) and maleic dialdehyde (MDA) were purchased from Sigma.

**Animals**

Sprague-Dawley (SD) rats (1-3 d old) were purchased from the Laboratory Animal Center of Sun Yat-Sen University.

**Neonatal mouse primary cardiomyocyte culture**

The procedure of culturing ventricular cardiomyocytes from neonatal mice was established by modifications of the previously described methods\(^7\)\(^-\)\(^10\). One- to three-day-old neonatal mice were euthanized by cervical dislocation. Hearts were removed aseptically from the mice, the ventricles were retained, and kept in Hank’s balanced salt solution (HBSS) without Ca\(^{2+}\) and Mg\(^{2+}\) (in g/L: 0.4 KCl, 0.06 KH\(_2\)PO\(_4\), 8.0 NaCl, 0.35 NaHCO\(_3\), and 0.06 Na\(_2\)HPO\(_4\); \(\mathrm{pH} 7.2\)) at 4 °C. The ventricles were washed thrice with HBSS and minced into small fragments and washed twice again during mincing. The cells were dissociated at 37 °C for 8 min in enzyme solution (0.8 g/L trypsin in HBSS without Ca\(^ {2+}\) and Mg\(^ {2+}\), \(\mathrm{pH} 7.2\)), the ventricle tissue was churned by the magnetic stirring (100 r/min) during the digestion. The cells released after the first digestion were discarded, whereas the cells after subsequent digestion were added to an equal volume of cold HBSS with Ca\(^ {2+}\) and Mg\(^ {2+}\) (in g/L: 0.14 CaCl\(_2\), 0.4 KCl, 0.06 KH\(_2\)PO\(_4\), 0.047 MgCl\(_2\), 8.0 NaCl, 0.35 NaHCO\(_3\), 0.049 MgSO\(_4\), 0.05 Na\(_2\)HPO\(_4\), and 1.0 D-glucose, \(\mathrm{pH} 7.4\)) until all cardiac cells were isolated (six times). The resulting mixture was centrifuged for 8 min at 200 r/min, and the cells were resuspended in the FBS-MEM. After being cultured for 48 h, the cells were transferred to different concentrations of H\(_2\)O\(_2\). Cardiomyocytes were plated at the density of \(1 \times 10^5\) cells/well containing 1 000 µL FBS-DMEM, and cultured in a water-saturated atmosphere of 50 mL/L CO\(_2\)-95% air box for 48 h. The myocardium was treated with 0.625, 1.25, 2.5, 5, 10 mmol/L H\(_2\)O\(_2\). A photo was taken to record the change of cardiomyocyte morphology after 3 h. The media were removed, the cells were collected and cell genome DNA was extracted, the change of genome DNA was analyzed and the process of cellular apoptosis was evaluated by electrophoresis on 1% agarose gels.

Cell viability was determined by short-term microculture MTT assay. On 96-well microplates, cardiac myocytes were plated at the density of \(3 \times 10^5\) cells/well containing 150 µL FBS-DMEM. After being cultured for 48 h, the cells were exposed to different concentrations (0.075, 0.15, 0.3, 0.6 mmol/L) of H\(_2\)O\(_2\) for 3 h, and then the media were replaced by 100 µL of DMEM and 20 µL of MTT solution (5 mg/mL). The cells were incubated for another 4 h. After being incubated for 4 h, the media and MTT solution were removed. The remaining formazan blue crystals were dissolved by MDSO. Absorbance at 570 nm was measured by Thermo Lab Systems (MultiSkran MK3).

**Cardioprotection of nm-haFGF**

The cardio-protective effect of nm-haFGF was evaluated on the basis of its anti-oxidative effect of nm-haFGF including measuring the total activity of SOD, monitoring the injury of cells membrane, examining cell viability as well as monitoring cell apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL).

SOD and MDA were measured by respective assay kit (Boster Co.). Cardiomyocytes were plated on 96-well microplates at the density of \(3 \times 10^4\) cells/well.
containing 100 μL FBS-DMEM, and cultured in a water-saturated atmosphere of 50 mL/L CO₂-95% air box for 48 h. The FBS-DMEM media were replaced by free-serum DMEM containing different concentrations of nm-haFGF (10, 20, 40, 80 ng/mL), and incubated in a water-saturated atmosphere of 50 mL/L CO₂-95% air for 24 h, then the cardiomyocytes were treated with 0.15 mmol/L H₂O₂. The leakage of MDA and the activity of SOD were measured after exposure to H₂O₂ for 3 h, and the cell viability of cardiomyocytes was measured by MTT.

TUNEL assay was performed. After being treated with nm-haFGF and H₂O₂, cardiomyocytes were fixed with 4% paraformaldehyde in PBS overnight at 4 °C. The sample was washed with PBS and then permeabilized by 0.2% Triton X-100 in PBS for 5 min at room temperature. After being washed, cells were equilibrated at room temperature for 5-10 min in equilibration buffer (200 mmol/L potassium cacodylate, 0.2 mmol/L dithiothreitol, 0.25 g/L bovine serum albumin, and 2.5 mmol/L cobalt chloride in 25 mmol/L Tris-HCl, pH 6.6) and then incubated in the presence of biotinylated nucleotide mix and terminal deoxynucleotidyl transferase at 37 °C for 1 h in a humidified chamber. The tail reaction was terminated by 2× standard saline citrate (SSC). Then, 0.3% hydrogen peroxide was added to block the endogenous peroxidase. After being washed thrice in PBS, streptavidin HRP was added for 30 min at room temperature, and washed in PBS again. At last, DAB was added to stain the sample, until a suitable background occurred and the positive cells were mounted under a reverse microscope. At least 1,000 cells were counted, and the percentage of TUNEL-positive cells was determined.

Statistical analysis
Data were expressed as mean±SD. Statistical analysis was performed by Student’s t-test to compare data in different groups. P<0.05 was considered statistically significant.

RESULTS

Neonatal mouse primary cardiomyocytes culture and identification
It was noted that the animal age should not be more than 3 d, because cardiomyocytes obtained from neonatal mice older than 3 d did not attach to the dishes. After being separated from non-muscle cells by preculturing for 2 h, the cardiomyocytes were replated at a density of 1.0×10⁵ cells/cm² and incubated under the condition described in Materials and methods. After being cultured for 24 h, almost all the cardiomyocytes attached to and spread on the substratum of the dishes and beat spontaneously. The culture media were changed at this time and then changed every 3 d. Forty-eight hours after culturing was completed, the myocytes were identified by the immunocytochemical assay. The cells isolated and cultured were cardiomyocytes, and the cellular morphological characteristics are shown in Figure 1. The myocyte purity averaged 94±2.25%.

Cytotoxicity of H₂O₂
Cardiomyocytes were exposed to different concentrations of H₂O₂. Morphological alteration, cardiomyocyte apoptosis and cell viability were measured as the cytotoxicity of this oxidant. As shown in Figure 2, a dramatic change of cellular morphology and a concentration-dependent injury effect could be observed. When exposed to 0.6125 mmol/L hydrogen peroxide for 3 h, there were no significant differences in cellular morphology. When the concentration increased, a dramatic change in cellular morphology was found. All the cells lost their characteristics of spontaneous beating, and cellular membrane became shrunk, cellular nuclei were contracted.

Genomic DNA was measured to indicate alterations in cardiomyocyte apoptosis, when cardiomyocytes were exposed to different concentrations of H₂O₂. As shown in Figure 3, genomic DNA remained intact after being exposed to 0.625 mmol/L H₂O₂ for 3 h; when H₂O₂ concentration was changed to 1.25 mmol/L, genomic DNA was divided into some small fragments; when H₂O₂ concentration increased to 5 and 10 mmol/L, all genomic DNAs were divided. There were electrophoresis stains, suggesting that apoptosis of cardiomyocytes occurred when they were exposed to H₂O₂.

MTT assay was used to monitor the cellular viability. There were significant differences in the cell viability at different concentrations of H₂O₂. The higher the concentration of H₂O₂, the lesser the viability of cardiomyocytes (Table 1).
Cardiomyocytes were exposed to a certain concentration of H\textsubscript{2}O\textsubscript{2} and different concentrations of nm-haFGF. The total activity of SOD, MDA leakage, cellular viability and apoptosis were measured as the parameters for evaluating the protective effects of nm-haFGF. As shown in Table 2, the total activity of SOD dramatically increased with the concentration of nm-haFGF, the MDA leakages decreased with the concentration of nm-haFGF, and there was a significant difference between the control and experiment groups. As shown in Table 3, cellular viability increased with the increasing of concentration of nm-haFGF. All these showed that nm-haFGF had a significant cardiomyocytes protective effect on cardiomyocytes exposed to H\textsubscript{2}O\textsubscript{2}.

To examine the effect of H\textsubscript{2}O\textsubscript{2} on apoptosis, myocytes were pretreated with different concentrations of H\textsubscript{2}O\textsubscript{2}. The initiating effect of H\textsubscript{2}O\textsubscript{2} on cardiomyocytes apoptosis was confirmed by determining the change of DNA fragment (Figure 3). The cellular protective effect of nm-haFGF was confirmed by the same method (Figure 4). The rate of TUNEL-positive cells was 32.9±3.3% in the H\textsubscript{2}O\textsubscript{2} group, and 1.1±0.26% in the normal group. But after being exposed to 0.15 mmol/L H\textsubscript{2}O\textsubscript{2}-DMEM, the rate of TUNEL-positive cells was 25.4±2.6% in 10 ng/mL nm-haFGF group, the cellular apoptosis rate in the 40 ng/mL nm-haFGF group was only 19.7±1.9%.

**DISCUSSION**

Studies indicate that intracoronary, intrapericardial or myocardial administration of FGFs, in chronically ischemic canine and porcine hearts, can stimulate angiogenesis, a process to minimize infarct size and improve cardiac function\cite{11-13}. Although there are some promising results in the animal model trials, some questions, such as dose dependence\cite{14}, acute negative inotropic effects\cite{10} still exist. Some investigators

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**Table 1** Viability of cardiomyocytes

| Group (n = 8) | Concentration of H\textsubscript{2}O\textsubscript{2} (mmol/L) | A\textsubscript{570 nm} (mean±SD) |
|--------------|-----------------------------|-------------------|
| Normal       | 0                           | 0.162±0.007       |
| 1            | 0.075                       | 0.153±0.016       |
| 2            | 0.15                        | 0.121±0.017\textsuperscript{b} |
| 3            | 0.3                         | 0.098±0.012\textsuperscript{b} |
| 4            | 0.6                         | 0.079±0.006\textsuperscript{d} |

\textsuperscript{a}P<0.05, \textsuperscript{b}P<0.01, \textsuperscript{c}P<0.001 vs normal group.

**Cardio-protective effect of nm-haFGF**

Cardiomyocytes were exposed to a certain concentration of H\textsubscript{2}O\textsubscript{2} and different concentrations of nm-haFGF. The total activity of SOD, MDA leakage, cellular viability and apoptosis were measured as the parameters for evaluating the protective effects of nm-haFGF. As shown in Table 2, the total activity of SOD dramatically increased with the concentration of nm-haFGF, the MDA leakages decreased with the concentration of nm-haFGF, and there was a significant difference between the control and experiment groups. As shown in Table 3, cellular viability increased with the increasing of concentration of nm-haFGF. All these showed that nm-haFGF had a significant cardiomyocytes protective effect on cardiomyocytes exposed to H\textsubscript{2}O\textsubscript{2}.

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wondered if there would be a potential danger of stimulating the growth of tumor for their strong power of mitogenesis[13]. In this study, we explored nm-haFGF responding to free radical-induced injury of primary cardiomyocytes when exposed to $H_2O_2$, which induces cardiac apoptosis[16,17]. As shown in Figure 2, when primary cultured neonatal SD mouse cardiomyocytes were exposed to different concentrations of hydrogen peroxide, a dramatic difference of morphological alteration was found, showing a concentration-dependent injury effect. The higher the concentration of $H_2O_2$, the more serious injury of the cardiomyocytes. As shown in Figure 3, at the lower concentration of $H_2O_2$, the cellular genomic DNA remained intact, when the concentration of $H_2O_2$ increased, cellular genomic DNA was divided into small fragments, suggesting that $H_2O_2$ has a strong cytotoxicity to primary cultured cardiac myocytes.

There is evidence that FGF family protein plays an important role in cardiac protection during hypoxia and reperfusion of ischemic myocardium[18-21]. It was reported that FGF should be considered as a protector rather than a promoter of myocardium regeneration in myocardium reperfusion injury[22-23].

In this study, we isolated and cultured the neonatal SD rat cardiomyocytes, built a cardiac myocyte injury model, and observed the cardio-protective effect of non-mitogenic human acidic FGF in vitro. We determined the cardiac myocytes injury by exposing it to different concentrations of $H_2O_2$, the cellular viability and the concentration of $H_2O_2$ showed a dose-dependent relationship. Cellular morphology and apoptosis examination showed that $H_2O_2$ had strong cytotoxicity to the cultured cardiomyocytes. nm-haFGF increased the total activity of SOD, protected the integrity of cellular membrane, and decreased the leakage of MDA as well as cell apoptosis, when cardiomyocytes were exposed to a certain concentration of $H_2O_2$. All these suggest that nm-haFGF can weaken the injury of cardiomyocytes in a dose-independent manner.

FGF has cardio-protective action on an isolated rat heart model[24-26]. Myocardial preconditioning can reduce infarct size, attenuate neutrophil infiltration, reduce calcium influx into cardiomyocytes, delay ultrastructural changes, and decrease programmed cell death (apoptosis)[23,28,29]. Therefore, one of the important mechanisms that can promote cardioprotection is by decreasing myocardial cell apoptosis identified in a wide variety of cardiovascular disorders, including myocardial infarction. As shown in Figure 3, when cardiomyocytes were exposed to a high concentration of $H_2O_2$, almost all the cells underwent apoptosis (Figure 2), and electrophoresis analysis showed the same results. When exposed to different concentrations of nm-haFGF, TUNEL assay showed that the rate of apoptosis dramatically decreased (Figure 4). All these indicate that nm-haFGF has the anti-apoptosis effect.

In conclusion, nm-haFGF has the anti-apoptosis effect at cell level.

ACKNOWLEDGMENT

The authors thank Dr. Tan Zhi (Physiological and Pathological Department, Sun Yat-Sen University) for his excellent technical support.

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