The Change and Significance of Proto-oncogene c-fos and Its Role in Viral Myocarditis

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Abstract **Objective** To study the change and significance of proto-oncogene c-fos in viral myocarditis(VMC). **Methods** The experiment established animal model of VMC by the way of coxsackie virus B3 inoculation, then our experiment studied the expression of protein and mRNA of proto-oncogene c-fos in VMC mice by ways of immunohistochemical analysis and in situ hybridization. At the same time, our experiment researched the significance of c-fos in VMC. **Results** The cardiomyocyte with positive expression of c-Fos protein increased apparently compared with control mice at 3 days after virus inoculation in VMC mice, which was almost normal at 35 days after virus inoculation. The expression level of c-fos mRNA was also higher than that in control group at 3, 7days after virus inoculation in VMC mice. **Conclusion** Expression of c-fos in cardiomyocyte increases in VMC mice, which may be related with the pathogenesis of VMC.

Key words Viral Myocarditis; Proto-oncogene; c-fos; Expression

The proto-oncogene c-fos participate in a variety of physiological process including cell growth, differentiation, transformation, signal transduction, plasticity of nervous function[1]. Its expression increase in some diseases and pathophysiology process, it may play a role in pathogenesis of some diseases. The report about the expression and function of c-fos in viral myocarditis (VMC) hasn´t been seen.Our experiment studied the expression and significance of mRNA and protein of proto-oncogene c-fos in VMC by ways of immunohistochemical analysis and in situ hybridization.

MATERIALS AND METHODS

Animals

BALB/c mice, male, 4~6 weeks old, 16~20g.

Main reagents

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Normal goat serum, Rabbit anti-c-Fos oncogene protein, Biotinylated goat anti rabbit IgG, Streptavidin Biotin-peroxidase Complex(SABC), antigen restoration solution, pepsin, c-fos oligonucleotide probe, Occlusive solution and rabbit anti digoxin were purchased from Boster Biological Technology Ltd. (Wuhan, China)and Sigma Chemical Co.(Sigma, St.Louis, MO).

Establishing of animal model(VMC)

130 mice were divided into two groups-experimental group (120mice) and control group (10 mice). Each mouse of experimental group was inoculated with coxsackie virus B3 (CVB3), and in control group the mice were inoculated with MEM Eagle´s solution 0.1ml. Mice of experimental group were killed at 3,5,7,9,15,35 days after inoculation.

Specimen collection

Serum was isolated from blood sample and was refrigerated. Each heart specimen was divided into two portions, one portion was fixed with 10% methanal, the paraffin-embedded tissue samples were cut into 5μm sections, the sections were stained using hematoxyline/eosin according to the standard procedures and observed under an Olympus light microscope, another portion

sample was preserved in liquid nitrogen(Will be used to the detection of RT−PCR) or was preserved with glutaric dialdehyde (Will be used as the sample of electron microscope).

Immunohistochemical analysis of c−Fos oncogone protein and in situ hybridization of c−fos oncogene.

Heart specimens were fixed with 10% methanal for 24 hours, the paraffin−embedded tissue samples were cut into 5μm sections, the sections were mounted on 3−aminopropyltriethoxysilane (APES) −treated slides, then were roasted (56℃ 1−2 hours, 37℃ 3 days) in oven.

Immunohistochemical analysis of c−Fos oncogene protein: After standard deparaffination and rehydration, the specimens were exposed to xylol for 10 minutes, 100% alcohol for 5 minutes, 96% alcohol for 5 minutes, and 70% alcohol for 3 minutes. The endogenous peroxidase activity was quenched by exposure to 3% hydrogen peroxide for 10 minutes. Antigen was restored by citrate−buffered(PH6.0). Normal goat serum was added for 10 minutes at room temperature, c−Fos antibody was added at 37℃ for 1.5 hours, then was rinsed with phosphate−buffered saline(PBS). Biotinylated goat anti−rabbit IgG was added for 20 minutes at 37℃, then was rinsed with PBS. Streptavidin Biotin−peroxidase Complex(SABC) was added for 20 minutes at 37℃, then rinsed with PBS. Color was then developed with diaminobenzidine (DAB) at room temperature, reactive time was controlled under light microscope, then was rinsed by distilled water. The sections were restained with hematoxylin, then were roasted at 37℃, sealing by neutral gum and was observed under light microscope.

In situ hybridization of c−fos oncogene: Formalin−fixed paraffin−embedded heart specimens were deparaffinized with xylene and rehydrated with graded ethanol. The endogenous peroxidase activity was quenched by exposure to 3% hydrogen peroxide for 10 minutes, then was incubated with pepsin(which was diluted with 3% citric acid) at 37℃ for 20 minutes, the sections were rinsed with 0.5M PBS and distilled water.

Digoxin−labeled probe was added to sections, then the sections were covered with coverslips overnight at 37℃, after the coverslips were disclosed, the sections were rinsed by 2×SSC (17.6g sodium chloride and 8.8g sodium citrate were added 1000 ml distilled water), and 0.2×SSC (1:10 dilution from 2×SSC). Rabbit anti−Digoxin was added to sections for 60 minutes at 37℃, then rinsed by 0.5M PBS. Biotinylated goat anti−rabbit IgG was added for 30 minutes at 37℃, then rinsed by 0.5M PBS. SABC was added for 30 minutes at 37℃, then rinsed by 0.5M PBS. The sections were coloured with DAB, reactive time was controled under light microscope. The sections were restained with hematoxylin, sealing by neutral gum and was observed under light microscope.

Determination of results

Blue cell nucleus were showed in normal cardiomyocyte, brown−yellow cell nucleus were showed in positive expression cardiomyocyte of c−Fos oncogene protein, brown−yellow particles were showed in positive expression cytoplasm of c−fos oncogene mRNA. The number of positive cell nucleus (or cytoplasm) of five high−power fields were calculated under light microscope, average value was calculated.

Statisticus

All data were expressed as mean±standard deviation (SD). *t* test or variance analysis were used to compare data between groups. A level of $P<0.05$ was considered statistically significant.

RESULTS

The expression of c−Fos oncogene protein in VMC mice

A few positive cardiomyocyte nucleus of c−Fos oncogene protein were seen in mice of control group. Positive cardiomyocyte nucleus of c−Fos protein expression increased apparently at 3 days after virus inoculation in VMC mice. The proportion of positive cardiomyocyte nucleus and total cardiomyocyte nucleus also increased apparently and increased further accompany with the advance of disease, peak level was at 7~9 days after virus inoculation(Table1, Figure1, 2). Positive cardiomyocyte nucleus of c−Fos protein expression were almost normal at 35 days after virus inoculation.

The expression change of c–fos oncogene mRNA

A few positive cardiomyocyte of c−fos mRNA expression were observed in mice of control group. Positive cadiomyocyte of c−fos mRNA expression increased apparently at 3 and 7 days after virus inoculation (Table2, Figure3).

DISCUSSION

Cells are triggered to change and grow by external signals, therefore proto−oncogenes figure heavily in signal transduction, a process that converts external stimuli into intracellular signals that guide cellular function. In the past 10~15 years of oncogene research, the identification of proto−oncogenes as specific components of signal transduction pathways has been the major discovery in the field. These and other more recent findings suggested that several new areas are emerging as important topics for future investigations in molecular oncogenesis.

Table 1 The expression change of c−Fos oncogene protein in VMC mice

Group	Number	PCN/HPF	PCN/TCN(%)
D_3	7	43.86±14.18△	9.52±2.80△
D_5	8	66.63±21.71△	16.73±5.76△
D_7	8	109.79±29.25△	27.92±7.87△
D_9	8	75.19±20.67△	18.26±4.71△
D_{15}	10	56.64±21.06△	13.62±5.08△
D_{35}	7	9.37±4.07	2.37±1.20
Control	10	8.25±2.44	2.03±0.60

PCN: Positive cardiomyocyte nucleus; TCN: Total cardiomyocyte nucleus

△: $P<0.01$compared with control group

Table 2 The expression change of c−fos oncogene mRNA in VMC mice

Group	Number	NPC/HPF	NPC/NTC(%)
D_3	7	28.22±10.31△	6.79±2.34△
D_7	8	52.24±16.69△	12.85±4.73△
Control	10	6.76±2.35	1.64±0.56

NPC: number of positive cardiomyocyte; NTC: number of total cardiomyocyte

△: $P<0.01$ compared with control group

Although the study of oncogene has provided some useful insights into cancer mechanisms, the most important benefit from oncogene research has been the delineation of the growth factor response pathway and molecular characterization of important cellular processes. The nuclear proto−oncogenes c−fos and c−jun have been particularly useful in this regard. Their study has provided important information about gene regulation in response to growth factors, regulation of immediate early genes, and the function and interaction of transcription factors.

The Fos oncogene was discovered as the cellular homologue of three distinct tumor viruses derived from mice and chickens [2]. Both the normal and viral Fos transforming proteins complex with a 39−KD protein[3]. The genes c−fos and c−myc were the first to be identified as immediate early genes after detailed analysis of their mRNA expression patterns. The transient induction of c−fos expression is mediated by multiple trans-acting factors. The c−fos mRNA and its 55−KD nuclear phosphoprotein (Fos) are rapidly but transiently induced by both growth factors and differentiating agents [4].

c−Fos may play a role as a potent inducer of apoptosis in pro−B cells and Ig class−switching B cells. c−Fos induced apoptosis is additionally supported by findings that induction of c−Fos expression is an early event in many instances of mammalian apoptosis [5,6] and that reduction of c−Fos activity by antisense oligonucleotides can prevent growth factor−deprived lymphoid cells from undergoing apoptosis. c−Fos may have a protective function, including DNA repair, against harmful consequences of agents[7].

The proto−oncogene c−fos encodes a nuclear phosphoprotein (c−Fos), c−Fos in a complex with products of another proto−oncogene, c−jun(AP−1), regulates the expression of AP−1 binding genes at the transcriptional level[8].

Over−expression of the proto−oncogene c−fos may play roles in some diseases, for example: Alzheimer´s disease, arthritis, myocardial stunning, neonatal hypoxia−ischemia,cardiac ischemia−reperfusion, heart failure,

and so on[9~18].

The c−fos protein expression induced by arthritis were found in rats, pathological pain following arthritis activated pain sensitive neurons and evoked c−fos expression in spinal cord. Over expression of c−Fos in central nervous system is induced by some pathological stimulation. c−fos mRNA was over−expressed in the hippocampal neurons of the patients with Alzheimer´s disease[9]. In rat models of myocardial stunning(MS), the expression of Fos protein increased apparently. It may play a role in MS, it may has relation with injury repair of molecular [10]. Cerebral hypoxia and/or ischaemia also produce hyperexpression of specific genes(c−fos, c−jun) which may be involved in the mechanisms of excitotoxic neuronal death. The expression of Fos being mainly associated with cellular damage and subsequent death following hypoxic−ischaemic in jury.

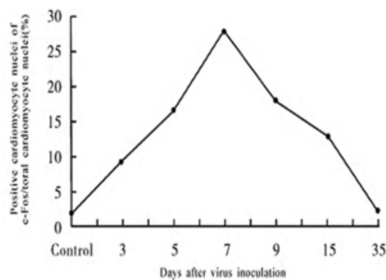

Fig. 1 The proportion change of positive cardiomyocyte nucleus of c−Fos protein in VMC mice

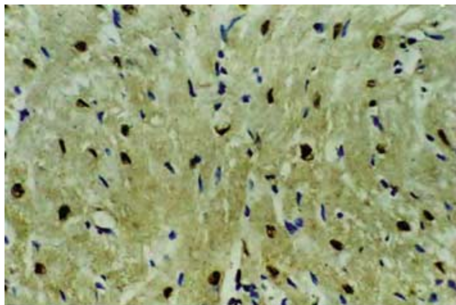

Fig. 2 The expression of c−Fos protein in cardiomyocyte of VMC mice at 9 days after virus inoculation(×400)

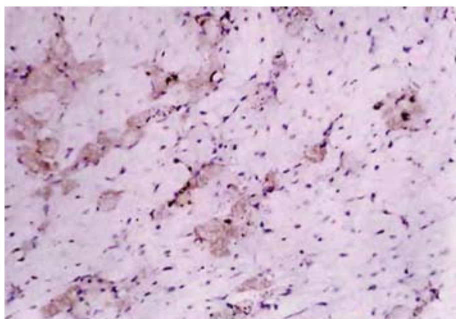

Fig. 3 The expression of c−fos mRNA in cardiomyocyte of VMC mice at 7 days after virus inoculation(×200)

Gonzalez CA *et al*[11] assayed Fos protein by the way of immunohistochemical staining, the results indicated that the administration of naloxone methiodide or naloxone to morphine−dependent rats induced marked Fos immunoreactivity within the cardiomyocyte nuclei western blot analysis revealed a peak expression of c−fos in the right and left ventricles after naloxone methiodide or naloxone−precipitaled withdrawal. Fos expression was increased after naloxone administration to morphine−dependent rats. These results suggested that the activation of c−fos expression observed during morphine withdrawal in the heart is due to intrinsic mechanisms outside the central nervous system (CNS). To analyze differential gene expression after myocardial ischemia−reperfusion, Nelson DP et al assayed the related immediate early genes c−fos and c−jun with northern analysis and in situ hybridization in human, and lamb myocardium subjected to cardiopulmonary bypass with myocardial ischemia. The results showed that c−fos and c−jun were induced in ischemia−reperfusion myocardium at endcardiopulmonary bypass. Expression patterns of c−fos and c−jun by in situ hybridization were markedly different; myocardial c−fos expression was diffuse and homogeneous, whereas c−jun expression was patchy with areas of intense focal localization [12].

Because TNF−α and other cytokines increase apparently in VMC [19~21], TNF−α and some other cytokines can induce the expression of c−fos and c−jun oncogene [22~25], so we deduced that abnormal expression of c−Fos can be observed in VMC. In our experiment, protein expression of c−Fos increased apparently compared with control mice at 3 days after virus inoculation, and increased further with the advance of disease, the top expression was at 7~9 days, and then decreased gradually and almost normal at 35 days after virus inoculation.

The expression of c−fos mRNA in VMC mice was also higher than that in control group apparently at 3 days and 7 days after virus inoculation. The results showe that the expression of c−fos increase in cardiomyocyte of VMC mice, c−Fos can compose AP−1 with c−jun gene products. TNF −α stimulated collagenase gene transcription, this stimulation is mediated by an element of the gene that is responsive to the transcription factor AP −1, and then the product of collagenase increase, collagenase play a important role in the course of tissue inflammation[26,27]. So we deduced that abnormal expression of c −fos maybe play a role in the inflammatory disease−−−VMC. In addition, c−fos also can regulate the transcription of relative gene of apoptosis, then regulate cardiomyocyte apoptosis indirectly [28] and thereby play a role in VMC. Because gene regulation mechanism of cell apoptosis is extremely complicate, c −fos may not play a main role in the regulation of cardiomyocyte apoptosis in VMC, and c−fos promote the transcription of collagenase gene possible mainly relate to its role in VMC.

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