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Cytosolic-Ca^{2+} and coxsackievirus B_3-induced apoptosis in cultured cardiomyocytes of rats

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KEY WORDS coxsackieviruses B; apoptosis; calcium; confocal microscopy; myocardium; cultured cells; electron microscopy

ABSTRACT

AIM: To explore the role of cytosolic free calcium ($[Ca^{2+}]_i$) in apoptosis induced by coxsackievirus B_3 (CVB_3) in cultured cardiomyocytes of rats. **METHODS**: Primary cultured cardiomyocyte was prepared from Wistar rats ages 2 – 3 d. The apoptosis in cardiomyocyte was determined by terminated deoxynucleotide transferase directed d-UTP nick and end labeling (TUNEL) method, and the apoptosis was observed under a transmission electron microscope. $[Ca^{2+}]_i$ in single cardiomyocyte loaded with Fluo 3-AM was measured by confocal microsorope. **RESULTS**: (1) The concentration of CVB_3 in the medium reached the peak at 24 h after CVB_3 infection. (2) The apoptotic cells were not found in CVB_3-infected cardiomyocyte in first 10 h, but amounted to 5 % at 17 h, 60 % at 24 h, and 90 % at 36 h. (3) The peak value of $[Ca^{2+}]_i$ elevation reached at 17 h after CVB_3 infection ($P < 0.01$). (4) The characteristics of apoptosis was also seen by transmission electron microscope. **CONCLUSION**: CVB_3 induced the apoptosis in cultured cardiomyocyte, and $[Ca^{2+}]_i$ mobilization was involved in the signal transduction process in apoptosis cells, and played an important role especially in the early stage of apoptosis induced by CVB_3.

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INTRODUCTION

Coxsackievirus B_3 (CVB_3), which belongs to small RNA virus and myocardiotropic virus, is an important factor in the pathogenesis of viral myocarditis. CVB_3 can directly cause myocardium damages resulting in cardiomyocyte death, also CVB_3 can activate specific cytotoxic T-cell (CTL) leading to the cardiomyocyte damage in resident by the existence in major histocompatibility complex (MHC). Recent study revealed that the apoptosis mechanism may be involved in the pathogenetic process of myocarditis[1,2] although the myocarditis by CVB_3 was mainly characterized by necrosis, and virus RNA was found continuously in cardiomyocyte after acute phase of myocarditis[3]. Ca^{2+}, an important second messenger, was involved in the regulation and metabolism in cytoplasm, gene transcription and expression, cell differentiation and reproduction, and also apoptosis. The present experiment attempted to demonstrate the apoptosis by CVB_3, and the role of cytosolic-free calcium during the apoptosis.

MATERIALS AND METHODS

Agents TUNEL testing box for apoptosis was purchased from Oncon Co. Fluo 3-acetoxymethyl (AM) ester (Molecular Probes Eugene OR, USA) was dissolved in Me_2SO 1 $g \cdot L^{-1}$ and stored at −20 ℃ in dark. Pluronic F-127 and HEPES were from Sigma.

Virus titration[4] According to Reed-Muench method, 50 % of tissue cultured infective dose ($TCID_{50}$, the number of $TCID_{50}$ per 0.1 mL) was determined in Hela cell at 0, 10, 17, 24, and 36 h after CVB_3 treated.

Preparation of cultured cardiomyocyte The hearts were taken from 2 – 3 d Wistar rats provided

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by Experimental Animal Center of Harbin Medical University (Grade Ⅱ, Certificate № 0921) and cut into pieces of about 1 mm^3 which were digested into cell suspension by 0.2 % collagenase. The single cardiomyocytes were got from centrifugation of cell suspension and resuspended with RPMI-1640 containing 20 % fetal calf serum (FCS). Cardiomyocytes were accounted for about 90 % after repeated purification. The cell suspension was adjusted into $5 \times 10^6 \cdot L^{-1}$ and plated on 25 mm round coverslip on the bottom of 6-well multidish for 48 h in CO_2 incubator, and then the cells were divided into normal group with RPMI-1640 containing 5 % FCS and CVB_3 infected group with RPMI-1640 + 5 % FCS containing 10^3 $TCID_{50}$ CVB_3 for 1.5 h. Both preparations were ready for apoptosis investigation and Fluo 3-AM loading.

Determination of apoptosis Cardiomyocyte apoptosis was investigated with TUNEL method[5] at 0, 10, 17, 24, and 36 h for the percentage of apoptosis cells. After that, the cardiomyocytes were stained by hematoxylin for the observation with light microscope.

Fluo 3-AM loading Cultured single cardiomyocyte lined on coverslip were washed twice with calcium-free PBS, and loaded in Fluo 3-AM 4 $\mu mol \cdot L^{-1}$ working solution containing 0.03 % F-127 at 37 ℃ for 40 min, and washing again with PBS containing $CaCl_2$ 1.5 $mmol \cdot L^{-1}$ to remove the extracellular Fluo 3-AM. This preparation was ready for the measurement of $[Ca^{2+}]_i$.

Measurement of $[Ca^{2+}]_i$ The coverslip-lined cultured cardiomyocytes loaded with Fluo 3-AM were mounted in the Auttofloul™ cell chamber (Molecular Probes) with 400 μL RPMI-1640 + 5 % FCS without phenolsulfonphthalein. The fluorescent intensity was detected by confocal microscope (Insight Plus-IQ, Meridian, MI, USA) with 40 × objective and 488 nm blue laser for excitation and 530 nm for emission at room temperature. The fluorescent images of $[Ca^{2+}]_i$ were collected at 0, 10, 17, and 24 h in both groups, and the $[Ca^{2+}]_i$ concentration change was represented by fluorescent intensity.

Preparation for transmission electron microscopy The cultured cardiomyocytes were digested by trypsin and collected into Eppendorff tube after washing. It was fixed by 2.5 % glutaraldehyde at 0 – 4 ℃ and washed by PBS 0.1 $mol \cdot L^{-1}$, fixed by osmic acid, washed by distilled water, and dehydrated by dimethylketone. After embedding with paraffin, the sample was cut into ultrathin sections (60 nm) for observation.

Statistic analysis Data were expressed as $\bar{x} \pm s$ and evaluated by t-test.

RESULTS

$TCID_{50}$ There was no CVB_3 infection in control group. At 24 h after CVB_3 infection, $TCID_{50}$ reached the maximum (Tab 1).

Tab 1. Apoptosis by TUNEL and $[Ca^{2+}]_i$ mobilization by CVB_3 in cultured cardiomyocytes ($n = 13 - 19$ from 8 rats), and $TCID_{50}$ in Hela cells. $[Ca^{2+}]_i$ was represented by fluorescent intensity (FI). $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* 0 h.

Time/h	$[Ca^{2+}]_i$/FI Control	CVB_3 treated	Apototic cells/%	$TCID_{50}$
0	970 ± 59	964 ± 46	0	0
10	984 ± 35[a]	1064 ± 214[a]	0	0.5
17	1008 ± 42[a]	2262 ± 382[c]	5.0	2.0
24	986 ± 72[a]	1386 ± 372[c]	60.0	4.77
36			90.0	4.23

Cardiomyocyte apoptosis After hematoxylin stain, the color of normal cardiomyocytes showed a light blue (TUNEL negative) and apoptosis cells (TUNEL positive) were light brown under the light microscope (Fig 1).

No apoptosis cells were found in control group. In CVB_3 treated group, apoptosis cells were observed at 17 h after CVB_3 infection, and reached the peak at 36 h (Tab 1).

$[Ca^{2+}]_i$ mobilization The fluorescent intensity (FI) of $[Ca^{2+}]_i$ showed no significant change within 24 h in control group. In CVB_3-treated group, the FI value of $[Ca^{2+}]_i$ was markedly increased ($P < 0.01$) and reached the peak (2262 ± 382, $P < 0.01$) from resting level (909 ± 26) at 17 h after CVB_3 treatment. Even after 24 h, the FI value of $[Ca^{2+}]_i$ was still maintained at a higher level than that in control group ($P < 0.05$). (Tab 1, Fig 2)

Morphologic changes With reversed light microscope, the normal cardiomyocytes were lined on coverslip with spindle or elliptic shape and had no

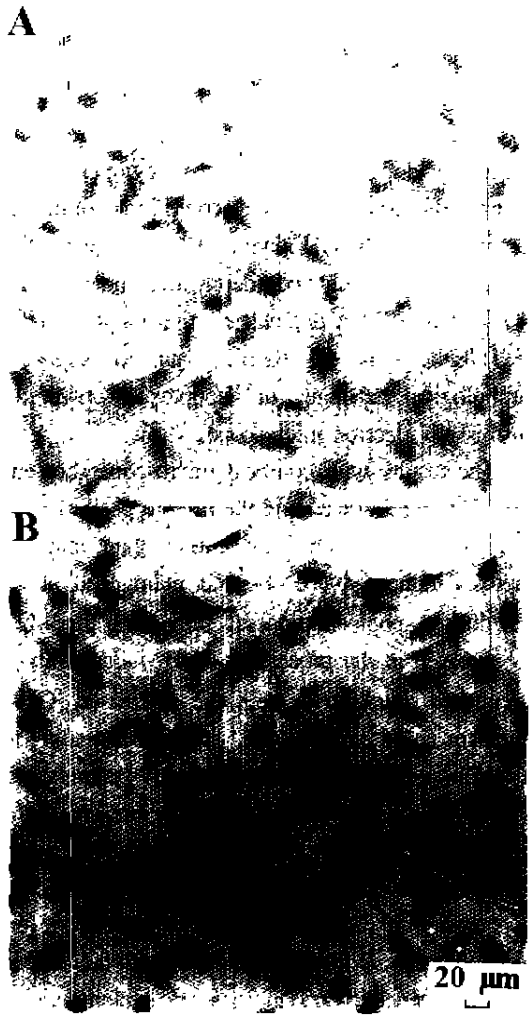

Fig 1. Cultured cardiomyocytes. Hematoxylin stain, ×132. A) Normal. B) treated with CVB_3 at 36 h.

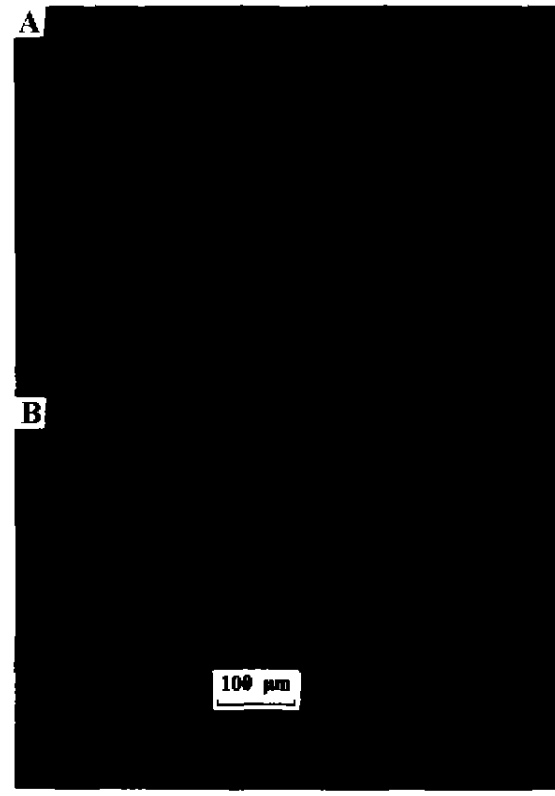

Fig 2. $[Ca^{2+}]_i$ mobilization in cultured cardiomyocytes before (A) and after (B) treatment with CVB_3 at 17 h.

obvious morphologic change within 17 h after CVB_3 infection, some cardiomyocyte changed into a round shape with higher refractivity and lost the connection with surrounding cells at 24 h, but still lined on coverslip. Up to 36 h after infection, the round cardiomyocyte suspended from coverslip and decreased in size.

With transmission electron microscope, the cardiomyocytes showed the characterized apoptosis, for example, the agglutination of chromatic border, cytoplasmic shrinkage, the reduction of nuclear size, mitochondria crista clear, and cell membrane integrity at 24 and 36 h in CVB_3-treated group. These morphologic changes were different from those in cytolysis of cultured cardiomyocytes which showed cell swelling with different sizes, even vacuolar degeneration, nuclear swelling, and fragmentation of nuclear membrane. (Fig 3)

DISCUSSION

Gene products from some RNA viruses, for example, the E_1A protein of adenovirus[6,7] and qp[3] of HIV[8,9], could induce the apoptosis of target cell. CVB_3 is a small RNA virus which usually caused the cytolysis and necrosis[10]. In our experiments, however, beside the cytolysis of myocardium in myocarditis of Balb/c model mice by CVB_3, the characteristic apoptosis cells were revealed by morphological observation. In cultured cardiomyocyte, CVB_3 also induced significant apoptosis without cytolytic change determined by TUNEL

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Fig 3. Transmission electronic microscope observation. A) Control (×12 000); B) apoptosis (×15 000); C) cytolysis (×9000) in cultured cardiomyocyte induced by CVB_3. a: agglutination of chromatic border, l: lysosome increase, m: vacuolar degeneration of mitochondria, n: nuclear swelling, s: cytoplasmic shrinkage, ↑: fragmentation of nuclear membrane.

method, suggesting that DNA fragmentation in apoptosis cardiomyocyte was prior to the necrosis characterized as cell membrane fragmentation resulting in cell organ swelling. In addition, because the CVB_3 RNA duplications were carried out in the smooth surfaced endoplasmic reticulium of cytoplasm, 3′-OH occurred during this process could not influence the measurement of 3′-OH due to DNA fragmentation in nucleus, and terminated deoxynucleotide transferase (TDT) was a DNA-dependent, the apoptosis revealed by TUENL was a reliable data to support the morphological change of apoptosis observed by transmission electron microscope.

The results with confocal microscope showed that $[Ca^{2+}]_i$ was mobilized and the time to peak of $[Ca^{2+}]_i$ mobilization was significant earlier than that of the apoptosis induced by CVB_3 and maintained at higher level during apoptosis process, suggesting that $[Ca^{2+}]_i$ was involved in the process of CVB_3-induced apoptosis in cultured cardiomyocyte. the increased Ca^{2+} in cytoplasm could elevated Ca^{2+} in nucleus leading to the activation and expression of some promoter genes involved in apoptosis, such as c-*myc* which was over-expressed in cultured cardiomyocyte by CVB_3, and the inhibition of the expression of survival genes, such as *Bcl*-2 gene[11,12], thus the activation of irreversible death process, for instance, the activation of endogenous nucleic endonuclease and glutaminase which can result in cell phenotypic change and cell death.

In summary, our results demonstrated that CVB_3 induced apoptosis in rat cultured cardiomyocyte, and Ca^{2+} was involved and played an important role in the early event of apoptosis.

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细胞内游离钙与柯萨奇病毒 B_3 诱导的大鼠培养心肌细胞凋亡

R965.2

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电镜

关键词 柯萨奇病毒 B；细胞凋亡；钙；共聚焦显微镜检查；心肌；培养的细胞；电子显微镜检查

目的：探讨$[Ca^{2+}]_i$在柯萨奇病毒(CV) B_3 诱导心肌培养细胞凋亡中的作用. **方法**：DNA 裂点检测法(3'-末端标记)及扫描电镜检测细胞凋亡. Fluo 3-AM 负载心肌细胞，共聚焦显微镜观察$[Ca^{2+}]_i$荧光强度变化. **结果**：感染 24 h，心肌细胞内 CVB_3 的浓度达峰值. 感染10 h未见凋亡的心肌细胞，17，24 和36 h凋亡细胞分别为5 %，60 % 和 90 %. 感染17 h心肌$[Ca^{2+}]_i$浓度达峰值. 电镜结果提示凋亡的特征. **结论**：CVB_3 可诱导培养心肌细胞的凋亡过程，细胞内 Ca^{2+} 参与凋亡细胞的信息转导过程，并在凋亡早期发挥重要作用.

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