·Basic Research·

# [Ca2+]i homeostasis and caspase-3 gene expression in verapamil-induced retinal pigment epithelium cells apoptosis *in vitro*

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## Abstract

• AIM: To study caspase-3 gene expression and [Ca2+]i homeostasis in verapamil (Ver)-induced human retinal pigment epithelium (RPE) cells apoptosis.

• METHODS: Ver 80mg/L was applied in cultured human RPE cells for 12, 24 and 48 hours to induce RPE cells apoptosis. The expression of apoptotic effector gene caspase-3 was assessed by reverse transcription polymerase chain reaction (RT-PCR). Single cell was measured using fluorescence indicator Fura-3/AM with MetaFluo4.5/coolsnapfx/IX70 intracellular $Ca^{2+}$ fluorescence imaging system.

• RESULTS: High levels of expression of caspase-3 mRNA were observed in normal RPE cells and it significantly increased after co-cultured with Ver. The fluorescence in resting RPE cells was strong and distributed throughout the cells. The nucleus appeared more fluorescent than the cytoplasm. Calcium fluorescence of RPE cells attenuated after co-cultured with Ver.

• CONCLUSION: Up-regulation of caspase-3 gene expression and disturbance of [Ca2+]i homeostasis might play pivotal roles in Ver-induced RPE cells apoptosis.

• KEYWORDS: [Ca2+]i homeostasis; caspase-3 gene; Ver-induced RPE cells apoptosis

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## INTRODUCTION

The retinal pigment epithelium (RPE) cell is a mosaic of polygonal cells interposed between the choroid and the neural retina and serves as the outer blood-retinal barrier regulating retinal homeostasis and visual function [1,2]. Normally RPE cells form acquiescent monolayer, but they retain the ability to divide and do so when placed in culture or when participating in wound repair [3]. After severe injury that may be associated with ocular trauma or retinal detachment, RPE cells can be detached and consequently found in the vitreous. Once in this new environment, RPE cells have been shown to dedifferentiate and exhibit a pseudometaplastic transformation into fibroblast-like, spindle-shaped cells, which become actively dividing and migratory. These processes are considered to be key events in the onset of proliferative vitreoretinopathy (PVR)[4]. PVR is an excess proliferation which ocular tissue is in response to repair in trauma, immigration and proliferation of RPE can promote PVR and RPE are the most important cells in forming proliferation membrane of PVR [5]. It is necessary to apply drugs or viteoretinal operation combined with drugs to inhibit the formation and development of PVR in the earlier period. We Previously applied calcium channel blocker-verapamil to induce cultured human RPE *in vitro* apoptosis successfully [6], but the mechanism of apoptosis and which apoptotic signals have involved in is unknown. Although it is widely accepted that intracellular calcium signaling and DNA damage might be the common triggers implicated in denomination of apoptosis, gene caspase-3 may play an important role in the executive phase of apoptosis. In this study, we further characterize human RPE cells apoptosis induced by Ver and explore whether caspase-3 gene expression and the calcium messenger system are involved in the regulation of verapamil (Ver)-induced cells apoptosis.

## MATERIALS AND METHODS

**Materials** Trizol agent, Super Script TM One-Step RT-PCR System was purchased from Takara. Caspase-3 primers (5'-TTGTGAAGTGCAAATGTTCTAAAGG-3', 5'-CAAGAAATCTCCCGTGAAATGTC-3'), and actin primers

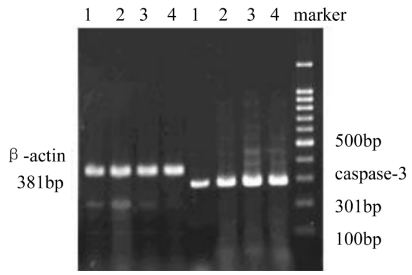


**Figure 1 Caspase-3 mRNA level in RPE cells treated with Ver**

**Table 1 The level of caspase-3 mRNA and $[Ca^{2+}]i$ of RPE cells in each group** (nmol/L,mean±SD)

| Groups | Caspase-3/ β -actin | $[Ca^{2+}]i$ |
|---|---|---|
| Control | 0.58±0.08 | 197.25±29.03 |
| 12 hours | 0.89±0.12[a] | 176.09±25.88[b] |
| 24 hours | 1.22±0.14[a] | 156.45±20.60[b] |
| 48 hours | 1.18±0.09[a] | 135.28±21.18[b] |
| *F* | 19.22 | 23.607 |
| *P* | 0.02 | 0.00 |

[a]$P<0.05$, [b]$P<0.01$ *vs* control

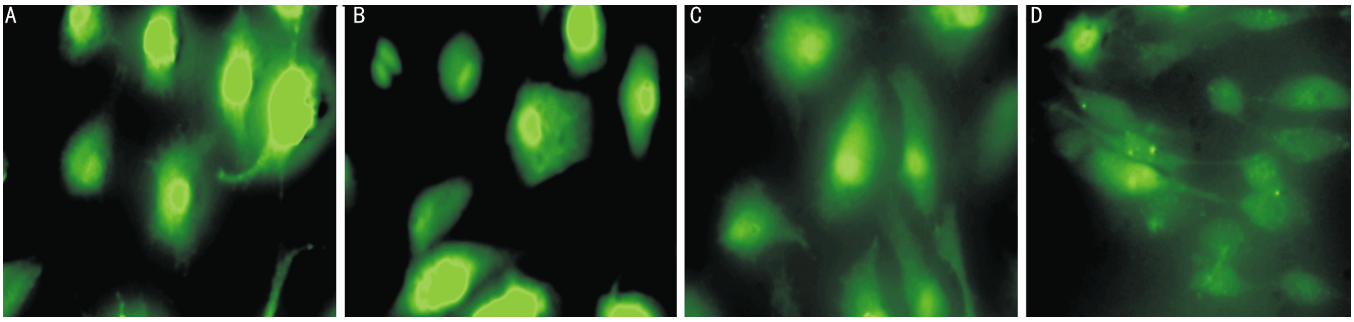


**Figure 2 Intracellular calcium fluorescence in control cultured RPE cells (Fluo-3/AM×400)** A: Control; B: 12 hours; C: 24 hours; D: 48 hours

(5'-AAATCGTGCGTGACATTAA-3', 5'-CTCGTCATACT CCTGCTTG-3') were obtained from Baoshengwu Co. Fura-3/AM was purchased from Eugene Oregon. Tissue source was obtained from the donator after the cornea transplantation from the department of ophthalmology in the first affiliated hospital of China Medical University. Their ages were between 24 and 38 years. Under sterile condition, using enzyme digestive method to isolate and complete plantation of human RPE cells by 2.5g/L trypsin to 24-pore culture plate. RPE cells were cultured in DEME containing 200mL/L fetal calf serum. Cultures were maintained at 37℃ in a humidified incubator under the atmosphere of 900mL/L air and 100mL/L $CO_2$.

**Methods**

**Caspase- 3 gene expression** Total RNA was extracted by Trizol agents. The following conditions were used for 35 cycles of PCR amplification: caspase-3: 30 seconds denaturation at 94℃, 30 seconds annealing at 56℃, and 2 minutes extension at 72℃. The amplified product was 301bp; actin: 30 seconds denaturation at 94℃, 30 seconds annealing at 63℃, and 2 minutes extension at 72℃. The amplified product was 381bp. The amplified products were resolved by gel electrophoresis on 20g/L agarose.

**$[Ca^{2+}]i$ in RPE cells** Planting the third period human RPE cells on 3.5cm$^2$ culture dish, applying 80mg/L Ver on cultured RPE cells for 12, 24 and 48 hours, control group was established simultaneously. After an overnight period of attachment, the medium was removed and the cells were loaded for 30 minutes at 37℃ with Fura-3/AM in PBS. The cells were then washed to remove extracellular Fura-3/AM and placed on MetaFluo4.5/coolsnapfx/-IX70 intracellular $Ca^{2+}$ fluorescence imaging system. Automatic running gel imaging system was used to scan the straps of PCR, optical density (OD) of each strap was read by FluorChen V.2.0 system. Calcium concentration of individual RPE cells was calculated by $[Ca^{2+}]i=Kd\ (F_{max}-F)/\ (F-F_{min})$, Kd=400, $F$ is fluorescence value.

**Statistical Analysis** Data were expressed as mean ±SD. Analysis of variance was adopted to conduct statistics test. $P<0.05$ was considered significant. Statistics was conducted by SPSS11.5 software.

**RESULTS**

**Caspase -3 expression** A low concentration of caspase-3 mRNA was detected in normal RPE cells and increased significantly after co-cultured with Ver for 12, 24 and 48 hours ($P<0.05$). Although the concentration of caspase-3 mRNA decreased a lot at 48 hours, it was still higher than that of normal RPE ($P<0.05$, Figure 1).

**$[Ca^{2+}]i$ in RPE cells** The fluorescence in RPE cells was strong and distributed throughout the cells. The nucleus appeared more fluorescent than the cytoplasm. Calcium fluorescence attenuated after cocultured with Ver ($P<0.01$, Figure 2,Table 1).

**DISCUSSION**

In recent years, there have been major insights into the mechanisms by which apoptosis is triggered in cells. The nuclear alterations, which are the pre-eminent ultrastructural changes of apoptosis, are often associated with

internucleosomal cleavage of DNA recognized as a "DNA ladder" on conventional agarose gel electrophoresis and long considered as a biochemical hallmark of apoptosis. However, internucleosomal cleavage of DNA appears to be a relatively late event in the apoptotic process, which in some models may be dissociated from early critical steps. Cell death protease designated as caspase may play an essential role in apoptotic cell death and act upstream of DNA fragmentation[7]. Caspases release pro-apoptotic factors known to serve as both signal transducers and effective components thus initiate a caspase cascade. Activation of caspase-8 has been shown to recruit downstream "amplifier" proteases, while caspase-3 is regarded as ultimately "effector" in the execution phase of apoptotic cell death [8]. Activation of caspases during apoptosis may result in the cleavage of critical cellular substrates, including poly (ADP-ribose) polymerase, so precipitating the dramatic morphological changes of apoptosis. Our results showed significant increase in the expression of caspase-3 gene following exposure to Ver, suggesting Ver-induced apoptosis in the RPE cells *via* caspase 3 dependent pathway. This is coherent with our previous results that Ver could down-regulate the expression of bcl-2 protein in the RPE cells [6]. Gene *bcl*-2, as the negative regulator of caspase-3, exerts anti-apoptosis action at or before the processing of certain caspases to their catalytically active forms[8]. Changes in [$Ca^{2+}$]i provide a chemical signal for early cell death pathway. If [$Ca^{2+}$]i can be elevated for a sustained period, cells are induced to undergo apoptosis [9]. But when [$Ca^{2+}$]i decreased, cells are also induced to undergo apoptosis. Homeostasis disorder of of calcium signaling system could be a mechanism of apoptosis[10]. Our experiment demonstrated that Ver elicited a sustained decrease of [$Ca^{2+}$]i which was a very early effect compared to morphological changes (cell rounding and shrinkage). Thus, we concluded that [$Ca^{2+}$]i might be another early initiator in connection with apoptosis. Although the precise mechanism needs further study, our findings provide the evidence for an important role of caspase-3 and homeostasis of calcium signaling system in the regulation of Ver-induced apoptosis.

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