

Chronic Graft Dysfunction and Improvement by Cytokine Response Modifier α Protein Transfection

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Abstract

Objectives: Cytokine response modifier α protein is a caspase inhibitor that inhibits caspase activity and protects cells from apoptosis. Chronic cyclosporine nephropathy is a significant cause of chronic graft dysfunction. We explored cytokine response modifier α protein-alleviated chronic cyclosporine nephropathy for ways of improving chronic graft dysfunction.

Materials and Methods: Cytokine response modifier α protein-transferring HK-2 cells were cultured with different concentrations of cyclosporine. Cytokine response modifier α protein mRNA and proteins were detected by real-time polymerase chain reaction and Western blot, cell viability was detected by (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and apoptosis was detected by flow cytometry.

Results: Cyclosporine caused a concentration-dependent and time-dependent loss of cell viability in HK-2 cells. Cytokine response modifier α protein mRNA was expressed at 48 and 72 hours ($P < .05$), while protein was detected at 72 hours. Cell viability in the cytokine response modifier α protein-transfected group was significantly greater than that of the control group when treated with 1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, or 20 $\mu\text{g/mL}$ cyclosporine at 24 or 48 hours ($P < .05$). The apoptosis in cytokine

response modifier α protein-transfected cells was significantly lower than that of controls ($P < .05$).
Conclusions: Cytokine response modifier α protein protects renal cells from cyclosporine injury by inhibiting activated caspases. Cytokine response modifier α protein transfection may improve chronic cyclosporine nephropathy and provide for improving chronic graft dysfunction.

Key words: *Crma* gene, Cyclosporine, Chronic graft dysfunction, HK-2 cell lines, Chronic cyclosporine nephropathy

Introduction

Chronic cyclosporine nephropathy is an important cause of chronic graft dysfunction. Long-term use of cyclosporine can cause chronic cyclosporine nephropathy by directly injuring renal cells.¹⁻² Cyclosporine could dramatically induce their apoptosis in time-dependent and dose-dependent manner when cocultured with different renal cells in vitro.³⁻⁵ When cyclosporine was given to Wistar rats and imprinting control region mice for 4 weeks, it induced chronic cyclosporine nephropathy that increased tubular and interstitial cell apoptosis in cyclosporine-treated animals.⁶⁻⁹

There were at least 6 apoptotic pathways that mediated renal cell apoptosis in vitro and in vivo. Cyclosporine induced renal cell apoptosis through Fas/FasL,¹⁰⁻¹¹ mitochondrial,^{2, 12} and the endoplasmic reticulum pathways.¹³ Cyclosporine also induced cell apoptosis through angiotensin 2,¹⁴⁻¹⁵ nitric oxide,¹⁵ and the hypertonicity-related apoptosis pathways.¹⁶ Activated caspases might be the final common pathway of all these pathways in vivo and in vitro. There were 21 interventions that could inhibit renal cell apoptosis induced by cyclosporine, such as antioxidant drugs,¹⁷⁻¹⁹

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recombinant human erythropoietin,²⁰ hepatocyte growth factor,²¹ spironolactone,²² rosiglitazone,¹⁴ and colchicine.²³ However, these interventions have not been clinically proven.

In 1986, Pickup and associates found that cytokine response modifier A protein has a 38 Kda protein virulence factor, which leads to hemorrhagic damage in the Brighton Red strain of cowpox viral infection.²⁴⁻²⁵ Cytokine response modifier A (CrmA) protein was a caspase inhibitor that broadly inhibits the activity of many caspases, such as caspase-3, caspase-4, caspase-5, caspase-8, caspase-9, and caspase-10, and then protects target cells from apoptosis induced by many factors such as immune injury, anoxia, and chemical factors.²⁶⁻²⁸

We hypothesized that CrmA-protected cyclosporine induced cell apoptosis and offer ideas for protecting graft kidney from injury by cyclosporine and improving chronic graft dysfunction.²⁹ In this article, the CrmA gene was transfected into HK-2 cells, and the CrmA protein inhibited activated caspases (their common pathway) and protected HK-2 cells from cyclosporine-induced apoptosis.

Materials and Methods

All protocols and experimental studies were approved by the ethics committee of the institution before the study began, and the protocol conforms to the ethical guidelines of the 1975 Helsinki Declaration.

Reagents

The human proximal tubular cell line HK-2 cells were obtained from American Type Culture Collection (American Type Culture Collection [ATCC], Manassas, VA USA), grown in Dulbecco Modified Eagle medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen) and HEPES free acid 15 mM, at 37°C, 95% air, and 5% CO₂. p-CAGGS-CrmA (LMBP3830) and p-CAGGS (LMBP-2453) were purchased from BCCM/LMBP plasmid collection MOSAICC (Micro-Organisms Sustainable use and Access regulation International Code of Conduct, Belgium). Lipofectamine 2000 was purchased from Invitrogen. Cowpox virus CrmA-Ab was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA) Cytokine response modifier A-Ab was

purchased from Santa Cruz Biotechnology, Inc. A Catrimo-14TM RNA Isolation Kit, a PrimeScript RT Reagent Kit (Perfect Real Time), and a SYBR Premix Ex Taq II were from Takara Biotechnology ([Dalian], Shiga, Japan). An Annexin V-FITC apoptosis detection kit was purchased from Calbiochem (San Diego, CA, USA). All other chemicals were of analytic reagent grade.

Cell culture

Human killer (HK)-2 cells were grown in 25-mm Falcon T flasks using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum. Transfection of the CrmA gene into HK-2 cells was achieved using Lipofectamine 2000 transfection agent; after its protocol, cell colonies were picked up and analyzed for expression of the CrmA protein and mRNA. Human killer-2 cells were harvested 72 hours after transfection and cultured with cyclosporine. Finally, their viability and apoptosis were detected by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Annexin V / propidium iodide (PI) staining assay.

Real-time polymerase chain reaction

We quantified mRNA levels of CrmA in HK-2 cells at 24, 48, and 72 hours after transfection (1×10^5) using a real-time fluorescence detection. RNAs were extracted from a single-cell suspension using the Catrimox-14 MIRNA Isolation Kit (Takara, Tokyo) according to the manufacturer's instructions. Real-time quantitative polymerase chain reaction experiments were performed with an multicolor real-time polymerase chain reaction detection system (Applied Biosystems, Foster City, CA, USA), using a SYBR Premix Ex Taq II according to the manufacturer's protocol. The primer sequences were as follows: for β -actin (sense, 5'-ATGGAGCCACCGATCCACA-3', and antisense, 5'-CATCCGTAAAGACCTCTATGCCAAC-3') and for CrmA (sense, 5'-TTCTCCACCGTCAATCTCGTC-3', and antisense 5'-CCTTATTCTTGTCGCCCTCCT-3'). Each sample was normalized based on its β -actin content. Thermal cycling conditions were as follows: 10 seconds at 95°C, followed by 40 cycles of 95°C for 5 seconds, and 60°C for 30 seconds.

Western blot analysis

At 72 hours after transfection, HK-2 cells were harvested and washed with cold phosphate buffered

saline and solubilized with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid [EDTA], 1 mM phenylmethanesulfonyl fluoride [PMSF], 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) at 4°C for 1 hour. Lysates were centrifuged at 14 000 revolutions per minute for 10 minutes, and the supernatant was collected as sample. Samples were separated on 14% SDS-polyacrylamide gel, and transferred to nitrocellulose membranes using standard electroblotting procedures. Membranes were blocked with 5% skim milk in Tris-Cl buffered saline (TBS-T, 0.1% Tween-20). Blots were incubated with primary antibodies (1:800) of CrmA at 4°C overnight. Immunoblots were washed, and then they were incubated with horseradish peroxidase conjugated secondary antibody (ZSbio, China) at room temperature for 1 hour and subsequently processed for enhanced chemiluminescence detection using BCIP/NBT alkaline phosphatase substrate solution protocol (Roche Pharmaceuticals, Basel, Switzerland). Signals were detected by a chemiluminescence detection system (Bio-Rad Laboratories, Clinical Diagnostics Group, Hercules, CA, USA).

MTT assay

Four hours before the end of the incubation, 20 μ L of 5 mg/mL MTT was added to each well. MTT was removed and replaced with 100 μ L of dimethyl sulfoxide (DMSO) for further incubation for 10 minutes at 37°C until the crystals were dissolved. The optical density value of each well was measured using a KCjunior Microplate Spectrophotometer (BioTek US, Winooski, VT, USA) with a test wavelength of 570 nm.

Human killer-2 cells were harvested, and the cells were divided into 24-hour and 48-hour groups. Concentrations of cyclosporine were 0.01 and 1000 μ g/mL. Each group had 5 repeated wells. Each well of a 96-well plate was seeded with 0.5×10^4 cells/200 μ L. Cells were incubated at 37°C under an atmosphere of 5% CO₂ for 24 or 48 hours. Cells viability was detected by MTT.

Human killer-2 cells were harvested 72 hours after transfection. Cells were divided into the following 3 groups: the p-CAGGS-CrmA group, the p-CAGGS group, and the nontransfected group. Each group had 5 repeated wells. Each well of a 96-well plate was seeded with 0.5×10^4 cells/200 μ L. Concentrations of cyclosporine were 1 μ g/mL, 10 μ g/mL, and

20 μ g/mL. Cells were incubated at 37°C under an atmosphere of 5% CO₂ for 24 or 48 hours. Cells viability was detected by MTT.

Annexin V/propidium iodide staining assay

HK-2 cells were harvested 72 hours after transfection. Cells were divided into the following 3 groups: the p-CAGGS-CrmA transfected group, the p-CAGGS transfected group, and the nontransfected group. Each group had 5 repeated wells. Each well of a 6-well plate was seeded with 1×10^5 cells/500 μ L. Concentrations of cyclosporine were 1 μ g/mL, 10 μ g/mL, or 20 μ g/mL. Apoptosis ratio was measured by BD FACSCalibur multicolor flow cytometer (Becton Dickinson, Sparks, MD, USA) according to the instruction provided by the annexin V/propidium iodide kit (BD Biosciences, San Jose, California, USA). Briefly, after treatment with cyclosporine 1 μ g/mL, 10 μ g/mL, and 20 μ g/mL for 24 hours, HK-2 cells were harvested and washed twice with precold phosphate buffered saline and resuspended in a binding buffer containing FITC-conjugated annexin V antibody (1.25 μ L) and propidium iodide, 10 μ L. After incubation for 30 minutes in the dark, cells were analyzed by flow cytometry.

Statistical Analyses

Statistical analyses were performed with SPSS software for Windows (Statistical Product and Service Solutions, version 13.0, SSPS Inc, Chicago, IL, USA). Differences in the means between each group were detected by the *t* test. Values for *P* < .05 were considered statistically significant.

Results

Quantitative analysis of cytokine response modifier A protein gene expression

Real-time quantitative polymerase chain reaction was used to quantify CrmA gene expression in the p-CAGGS-CrmA transfected HK-2 cells, p-CAGGS transfected HK-2 cells, and nontransfected HK-2 cells. Amplification specificity for CrmA and β -actin was determined by analyzing the dissociation curves. Cytokine response modifier A protein expression was detected in CrmA transfected HK-2 cells (*P* < .05), not in p-CAGGS transfected or nontransfected HK-2 cells (*P* < .05). The highest level of CrmA expression was detected in CrmA transfected HK-2 cells at 72 hours (*P* < .05) (Figure 1).

Expression of cytokine response modifier A protein in human killer-2 cell lines

To test expression of CrmA protein in transfected HK-2 cells, we detected cell transfection after 72 hours. In Figure 2, Western blot analysis demonstrated that CrmA expression (34-48 Kd) was seen in CrmA transfected HK-2 cell lines. Cytokine response modifier A protein expression was not found in the p-CAGGS transfected group or nontransfected group.

Drug toxicity of cyclosporine to human killer-2 cell

Human killer-2 cells were cocultured with different doses of cyclosporine. In Figure 3, cyclosporine directly damaged HK-2 cells; its drug toxicity also was increased with dosage and time, and the corresponding cell activity was decreased. Cell viability of HK-2 cocultured with cyclosporine (0.01-10 $\mu\text{g/mL}$) at 48 hours was higher than it was at 24 hours. When HK-2 cells were cocultured with cyclosporine (> 200 $\mu\text{g/mL}$) at 24 or 48 hours, their activity was completely lost.

Protection of human killer-2 cell viability by cytokine response modifier A protein

At 72 hours after transfection, each cell group (the CrmA-transfected group, the p-CAGGS transfected, and the nontransfected group) was treated with 1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, or 20 $\mu\text{g/mL}$ of cyclosporine for

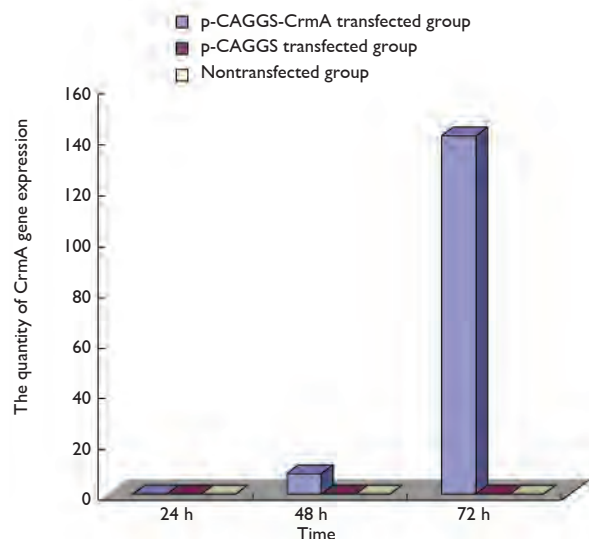


Figure 1. The real-time analysis of the amount of gene transcript relative to actin transcript in p-CAGGS-CrmA transfected HK-2 cells, p-CAGGS transfected HK-2 cells and nontransfected HK-2 cells. Comparison of the level of mRNA (relative to actin mRNA) among different time points was performed by the *t* test; All the data were analyzed from 4 individuals. Vertical bars represented the mean $\pm$ SD ($n = 4$). 72 h vs 48 h ($P < .05$); 72 h vs 24 h ($P < .05$); 48 h vs 24 h ($P < .05$).

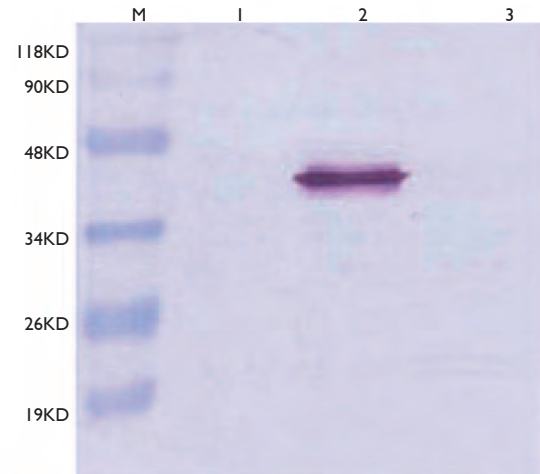


Figure 2. Western blot analysis of CrmA protein levels, (1) p-CAGGS transfected group, (2) p-CAGGS-CrmA transfected group; (3) nontransfected group, Fermentas: 118, 90, 48, 34, 26, 19kD.

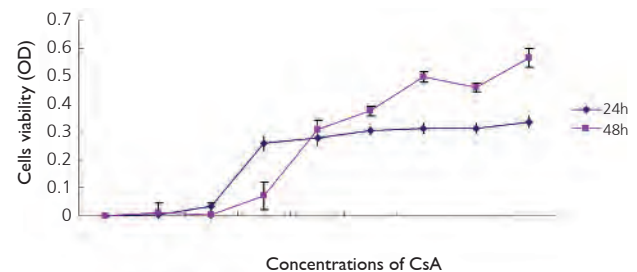


Figure 3. Drug toxicity of CsA to HK-2 cell

24 or 48 hours. Then the MTT assay was done to quantify protection of HK-2 cells by CrmA. Optical density value was used to represent cell viability. As shown in Figure 4, the cell viability in the p-CAGGS-CrmA transfected group was significantly higher than that of the other groups treated with 1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, or 20 $\mu\text{g/mL}$ of cyclosporine at 24 or 48 hours ($P < .05$). Cyclosporine could significantly decrease HK-2 cell viability in a concentration-dependent manner in controls (p-CAGGS transfected and nontransfected group) ($P < .05$). There was no significant difference in cell viability in either the p-CAGGS transfected group or the nontransfected group ($P > .05$).

Protection of HK-2 cell apoptosis by cytokine response modifier A protein

Detection of apoptosis of HK-2 treated with cyclosporine is usually characterized by the phosphatidylserine exposure at the outer leaflet of the plasma membrane. Based on their affinity for annexin V, apoptotic cells can be distinguished from annexin V-negative living cells by cytometric

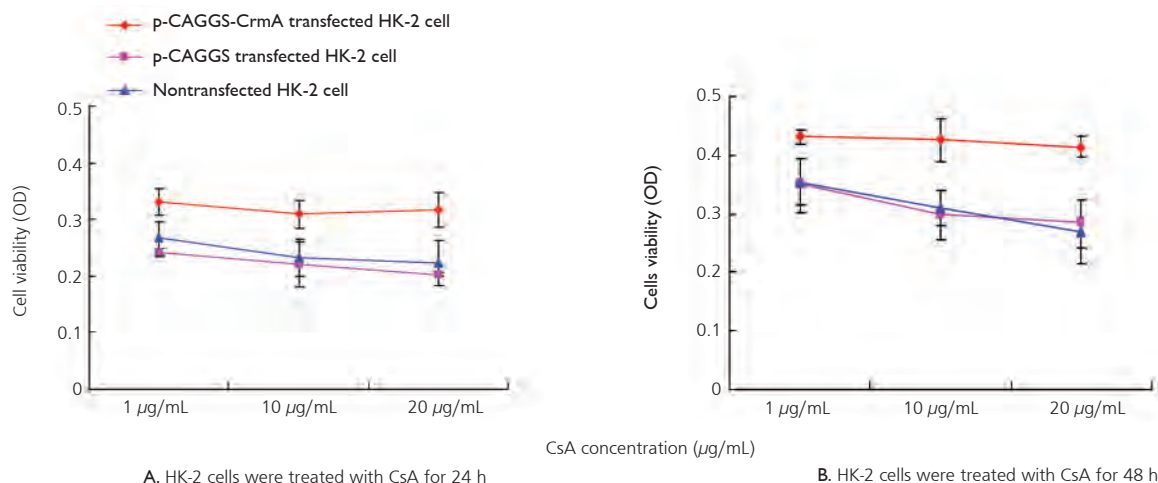


Figure 4. Protection of cell viability by CrmA in HK-2 cells. HK-2 cells were transfected with p-CAGGS-CrmA or p-CAGGS for 72 h. And then they were treated with CsA of 1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$ or 20 $\mu\text{g/mL}$ for 24 h or 48 h. Cell viability was assessed by MTT assay (p-CAGGS-CrmA transfected group vs controls ($P < .05$), p-CAGGS transfected group vs nontransfected group ($P > .05$)) in 24 h or 48 h.

procedures.³⁰⁻³¹ Furthermore, the double labeling assay (annexin V combined with propidium iodide) allows further distinction of necrotic or late apoptotic (annexin V+ / PI+) and early apoptotic cells (annexin V+ / PI-).

As shown in Figure 5, flow counting cells were 18605.7 ± 2461.11 . Cyclosporine could induce apoptosis in cells of varying degrees (including early and late apoptosis). The apoptosis rate in CrmA transfected cells was significantly lower than it was in controls (the p-CAGGS transfected and the nontransfected group) ($P < .05$). It induced HK-2 cells apoptosis in a concentration-dependent manner in the control groups ($P < .05$). The rate of apoptosis showed no significant difference in the p-CAGGS transfected group or the nontransfected group ($P > .05$).

Discussion

Cyclosporine is widely used in organ transplant and autoimmune diseases,³² but it induces kidney cell apoptosis through many apoptotic pathways, causes chronic cyclosporine nephrotoxicity, and has become an important cause of chronic graft dysfunction.³³⁻³⁸ We hypothesized that CrmA might protect cyclosporine-induced cells from dying and provide new ideas for protecting graft kidney from injury by cyclosporine and improving chronic graft dysfunction. Cytokine response modifier-A protein gene was transfected into HK-2 cells, and the CrmA protein inhibited the activated caspases (their common pathway) and protected the HK-2 cells from apoptosis induced by cyclosporine.

Previous studies have shown that when cyclosporine of different concentrations is cocultured with renal tubular epithelial cells of human, pig, dog, and mouse, it could significantly induce apoptosis in a dose-dependent and time-dependent manner.^{5, 10, 39-41} Here, we confirmed that cyclosporine caused a concentration-dependent and time-dependent loss of cell viability in HK-2 cells. Cell viability was recovery at 0.01 to 10 $\mu\text{g/mL}$ of cyclosporine when the coculture time was extended from 24 to 48 hours. When HK-2 cells cocultured with cyclosporine ($> 200 \mu\text{g/mL}$) at 24 or 48 hours, their activity was completely lost. Therefore, we speculate that the injury was reversible under 10 $\mu\text{g/mL}$ of cyclosporine, though it was not at 10 $\mu\text{g/mL}$ of cyclosporine and more.

Regarding clinical use of drugs, plasma concentration must be controlled in safe and effective dosage range. There have been studies that cyclosporine nephrotoxicity can be restored after withdrawal of cyclosporine.⁴²⁻⁴³ However, this does not explain the reversible phenomenon of low-dose cyclosporine nephrotoxicity.

The p-CAGGS-CrmA was transfected into the HK-2 cells. Cytokine response modifier A protein mRNA was detected at 48 and 72 hours and reached its peak at 72 hours. Cytokine response modifier A protein was detected by Western blotting at 72 hours. This result shows that p-CAGGS-CrmA is transfected and expressed in HK-2 cells by Lipofectamine 2000. Cytokine response modifier A protein was a caspase inhibitor and broadly inhibited the activity of many caspases.

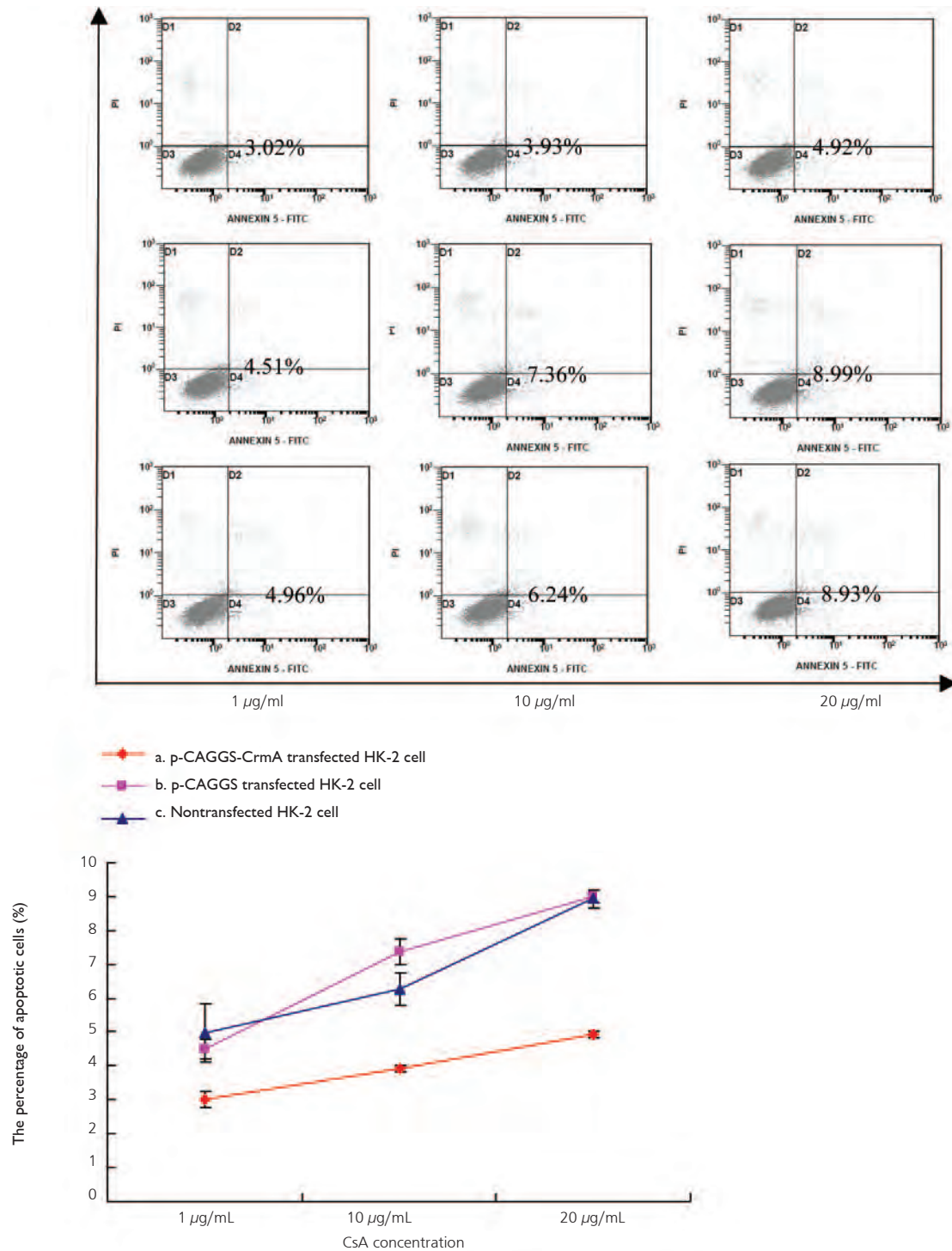


Figure 5. Apoptosis induced by CsA. The horizontal axis was AnnexinV-FITC and vertical axis was propidium iodide (PI). Apoptotic cells were D2 and D4. HK-2 cells were exposed to CsA of 1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$ or 20 $\mu\text{g/mL}$ for 48 h (A). The percentage of apoptotic cells treated with CsA of various concentrations (B). (p-CAGGS-CrmA transfected group vs controls ($P < .05$), p-CAGGS transfected group vs nontransfected group ($P > .05$)).

In this study, each group of cells (the p-CAGGS-CrmA group, the p-CAGGS group, and the nontransfected group) at 72 hours after transfection was treated with 1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, or 20 $\mu\text{g/mL}$ of cyclosporine for 24 or 48 hours. We found that the HK-2 activity in CrmA-transfected group was

significantly higher than in controls by MTT3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay ($P < .05$). The apoptosis rate in CrmA-transfected group was significantly lower than it was in the control groups by fetal calf serum ($P < .05$). Cell activity and the rate of apoptosis were no different in

the p-CAGGS-transfected group and nontransfected group ($P < .05$).

Our results indicate that CrmA has different degrees of protecting HK-2 cells from injury induced by cyclosporine at levels of 1 $\mu\text{g}/\text{mL}$, 10 $\mu\text{g}/\text{mL}$, and 20 $\mu\text{g}/\text{mL}$. Related studies have indicated that high expression of CrmA proteins in target cells inhibit cell apoptosis and protect them from injury induced by many chemicals, for example, cisplatin,⁴⁴ paclitaxel,⁴⁵ parthenolide,⁴⁶ and resveratrol.⁴⁷ Other studies show that target organ cells with high expression of CrmA are resistant to specific cytotoxic T lymphocyte,⁴⁸ inflammatory cytokines,⁴⁹ and hypoxic injury.⁵⁰ Adachi and associates found that CrmA gene transfection could significantly prolong the survival of a rat liver graft by DA-LEW orthotopic liver transplant.⁵¹⁻⁵²

In our study, we speculated that CrmA shows high expression in graft transplant and also can inhibit graft rejection, ischemia reperfusion injury, and cyclosporine nephrotoxicity; it can induce comprehensive tolerance of target organs and improve chronic graft dysfunction. These results will be confirmed in further studies.

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