

## Cyclosporine A Induces Apoptosis of Human Lung Adenocarcinoma Cells *via* Caspase-dependent Pathway

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**Abstract.** *Background: Aerosolized cyclosporine A (CsA) increases the local concentration of CsA in lung tissue and has proven to be an effective therapy for refractory rejection in lung transplant patients. However, the safety of high concentrations of CsA on tumour progression remains controversial. Materials and Methods: Human lung adenocarcinoma A549 cells were cultured with or without 1-3 µg/ml of CsA. The percentage of apoptotic cells was evaluated by Annexin V staining. The expressions of caspase-3, -9, -8 and cytochrome c were determined by Western blotting. Results: CsA therapy suppressed the growth of human lung cancer cells and increased the percentage of apoptotic cells compared with control cells. Western blot analysis revealed that CsA increased the levels of cytosolic cytochrome c and cleaved caspase-3 and -9, but not of cleaved caspase-8 in the lung cancer cells, suggesting that CsA-induced apoptosis is associated with the activation of caspase-3 and -9. Conclusion: Our findings indicate that a high concentration of CsA has cytotoxic effects through the caspase-3- and -9-dependent apoptotic pathway. This result shows that local administration of CsA does not increase the risk of secondary lung cancer.*

Lung transplantation has evolved as a life-saving alternative for many patients with advanced lung diseases, such as chronic obstructive pulmonary disease, cystic fibrosis, interstitial lung diseases, and other conditions that result in permanent lung dysfunction. In the United States, the number of lung transplants performed increased from 93 in 1989 to approximately 1500 in 2008 (1). In Japan, a total of 105 lung transplantations had been performed by the end of 2007 (2).

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Rejection of the transplanted organ remains a persistent problem for lung transplant recipients manifesting in both acute and chronic forms. Cyclosporine A (CsA) is an immunosuppressant widely used for suppressing rejection of organ transplants and in treating autoimmune diseases. Its mechanism of immunosuppressive action involves inhibition of T-lymphocyte calcineurin, cytokine production and immune cell activation.

Systemic administration of CsA incurs the risk of end-organ toxicity. The increased incidence of infections, nephrotoxicity, diabetes, hypertension and malignancies among the transplant population as a result of intense immunosuppressive therapy is well documented (3). Regional immunosuppression offers an approach to address both of these issues simultaneously by achieving high local drug levels with concomitant low systemic levels. Various experimental models of heart, liver and kidney transplantation have been used to examine the efficacy of the local delivery of aerosolized CsA. These studies report promising results in controlling rejection while maintaining low systemic drug concentration through local drug delivery.

The development of aerosolized CsA is directed specifically to the needs of lung transplant recipients, because the allograft is accessible for therapy by the inhaled route. We believe that local application of the drug would allow for treatment or prophylaxis of lung transplant rejection without increased rates of nephrotoxicity, and that the implementation of the drug might allow for reductions in other immunosuppressive medications.

In animal models of lung transplantation, aerosolized CsA remains in high concentrations in the lung tissue with low systemic concentrations of CsA. It is implying that aerosolized CsA has high efficacy for local immunosuppression and low potential for systemic side effects (4, 5).

In general, immunosuppressive drug therapy using CsA increases long-term survival rates after organ transplantation. But there are cases of patients with life-threatening complications such as cancer opting for transplantation. For example, lung transplant recipients are at risk of harboring

or developing bronchogenic carcinoma in their native lungs (6, 7). It has been reported that bronchogenic carcinoma develops in the native lung of transplant recipients with emphysema and pulmonary fibrosis, at frequencies of 2% and 4%, respectively (8).

Several reports indicate that CsA induces apoptotic cell death in leukemia, melanoma and glioma cells (9-15). In contrast, a previous report revealed that CsA accelerates tumour growth in lung cancer (16). Therefore, the safety of CsA usage on tumour progression remains controversial. Furthermore, whether the high concentration of CsA achieved by inhalation therapy has an effect on lung cancer progression remains unknown.

## Materials and Methods

**Cell line and reagents.** Lung adenocarcinoma cell line A549, purchased from the American Type Culture Collection (Rockville, MD, USA), was cultured in RPMI-1640 medium with 10% fetal calf serum (FCS) at 37°C in 5% CO<sub>2</sub>. Cells in the logarithmic growth phase were used. CsA was purchased from Sigma Chemical Company (St. Louis, MO, USA). An Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (San Diego, CA, USA). A mitochondria/cytosol fractionation kit was purchased from BioVision (Palo Alto, CA, USA). Antibodies for caspase-3, -9 and -8, and cytochrome c were purchased from Cell Signaling Technology (Beverly, MA, USA).

**In vitro clonogenic assay.** Clonogenic assay was used to determine the cytotoxic effects of CsA. A549 cells ( $4 \times 10^2$  to  $\times 10^4$  cells) were cultured in 1.72% methylcellulose medium containing 20% FCS (35 mm dish) at 37°C in 5% CO<sub>2</sub>. CsA at 1.0, 2.0 and 3.0 µg/ml concentrations was added to the medium. Following 7 days of incubation, the number of colonies containing more than 30 cells was counted by microscopy. The survival fraction was calculated by dividing the number of colonies obtained in the presence of CsA by the number of colonies obtained in vehicle treatment. The results were used to determine the cytotoxic effects of CsA.

**Detection of apoptosis by flow cytometry.** A549 cells were plated in 96-well tissue-culture plates in RPMI/FCS. After 24 h the cells were exposed to the CsA concentrations 0.5, 1.0 and 2.0 µg/ml, and cultured for 0, 24, 48, 72 and 96 h. At each time point, A549 cells ( $1 \times 10^6$  cells) were washed with phosphate buffered saline (PBS) and suspended in binding buffer in a polystyrene test tube. FITC-conjugated Annexin V and propidium iodide (PI) were added to the suspension, incubated at room temperature for 15 min, and the cell fluorescence was then determined by flow cytometry.

**Detection of apoptosis by Western blotting.** A549 cells were exposed to 1.0 µg/ml CsA and cultured for 0, 24, 48, 72 and 96 h. At each time point, the cells were washed with PBS and lysed in Tris-HCl buffer containing phenylmethylsulfonyl fluoride (PMSF), aprotinin and dithiothreitol (DTT). The lysates were centrifuged, and the supernatants were used as protein samples. The samples were boiled with sodium dodecyl sulfate (SDS) sample buffer for 3 min, separated by SDS (15%)-polyacrylamide gel electrophoresis and then electrotransferred to polyvinylidene difluoride membranes.

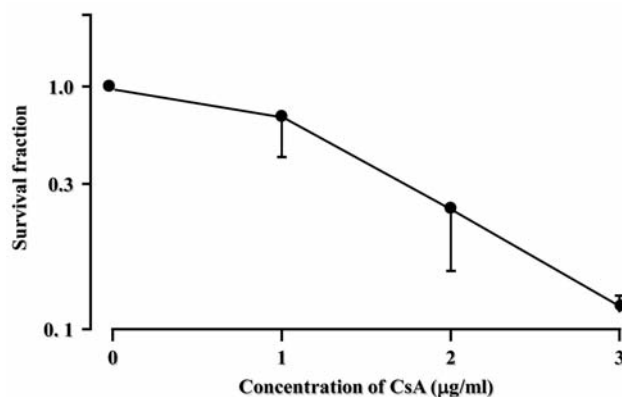


Figure 1. Cytocidal effect of CsA on A549 cells. A549 cells were treated with three different concentrations of CsA, the survival fractions measured following 7 days incubation. Data are the mean SD of average values obtained from three separate experiments performed on different days.

The membranes were blocked with 5% non-fat milk in TBS-T (Tris-buffered solution/0.1% Tween 20), incubated overnight with 1:1000 diluted primary antibodies, washed with TBS-T and further incubated for 1 h with 1:2000 diluted secondary antibodies (horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgM). The membranes were then immersed in color development solution and developed. Primary antibodies used for Western blotting were anti-caspase (caspase-3, -9 and -8) and cytochrome c. For cytochrome c, the mitochondria/cytosol fractionation kit was used to separate the cytosol and the mitochondrial protein fractions, following which the measurements were taken.

## Results

**Cytocidal effects of CsA.** We first examined the cytotoxic effects of CsA on A549 cells using clonogenic assay. As shown in Figure 1, CsA therapy reduced the survival fraction of A549 cells in a dose-dependent manner. Survival fraction values were 0.730, 0.360 and 0.131 at CsA concentrations of 1.0, 2.0 and 3.0 µg/ml, respectively. These findings demonstrate that CsA has a cytotoxic effect on A549 cells.

**Apoptotic effect of CsA.** To examine whether CsA-inducing cytotoxicity was due to apoptosis, FACS analysis using Annexin V with PI was performed. A549 cells were treated with 1 µg/ml CsA. The percentage of apoptotic cells increased to 17.5% at 72 h and 33.4% at 96 h in CsA-treated cells (Figure 2, middle panel) as compared with 8.3% at 72 h and 14.9% at 96 h in vehicle-treated cells (Figure 2, upper panel). These findings show that CsA induces apoptosis of A549 cells. The findings for Annexin V were also validated with H<sub>2</sub>O<sub>2</sub> as a positive control (Figure 2, lower panel).

**Measurement of apoptosis-related proteins by Western blotting.** Cytochrome c release from mitochondria to cytosol:

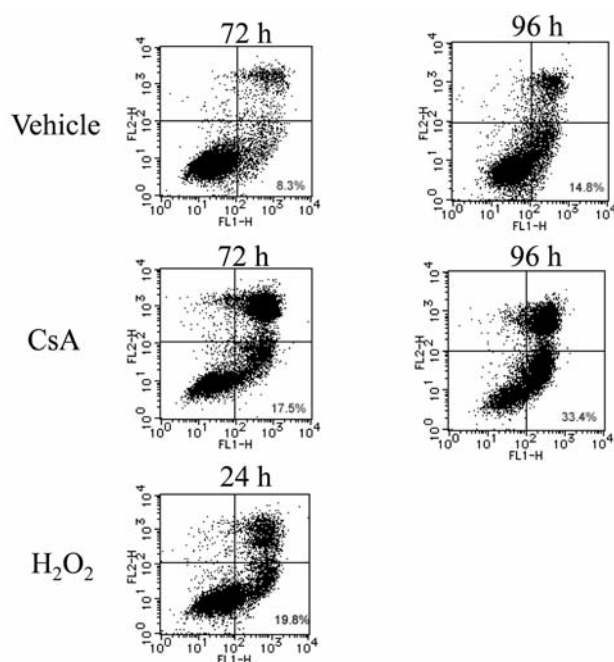


Figure 2. Apoptotic effect of CsA on A549 cells. Apoptosis was evaluated after treating A549 cells with vehicle control (upper panel), 1  $\mu$ g/ml CsA (middle panel), or 1 mM H<sub>2</sub>O<sub>2</sub> (lower panel) then staining with Annexin V (FL1-H) and PI (FL2-H) at 72 h and 96 h. The percentage of early apoptotic cells under each condition is provided in the lower right quadrant of the figure.

Cytochrome c release from the mitochondria is a critical step in the apoptotic cascade, because it activates downstream caspases. To examine the release of cytochrome c in CsA-treated A549 cells, we conducted Western blotting of the cytosol fraction. The findings demonstrate that CsA increased cytosolic cytochrome c in a time-dependent manner (Figure 3).

**Activation of caspase-9 and -3 in CsA treatment.** Caspases are known to play a central role in various apoptotic responses, including mitochondrial-mediated apoptosis. To identify the apoptotic pathway, we evaluated the roles of caspase-3, 9 and 8 in CsA-induced apoptosis. Western blot analysis revealed that the level of caspase-9 precursor was reduced by CsA therapy in a time-dependent manner. In contrast, the level of cleaved caspase-9 was increased (Figure 4). However, we observed no significant change in the level of pro-caspase-8 in CsA-treated A549 cells (data not shown). We found that CsA significantly induced pro-caspase-3 cleavage to its active form in a time-dependent manner as can be interpreted from the Western blot analysis result (Figure 5). These results suggest that CsA-induced apoptosis is associated with the activation of caspase-9 and -3, but not of caspase-8.

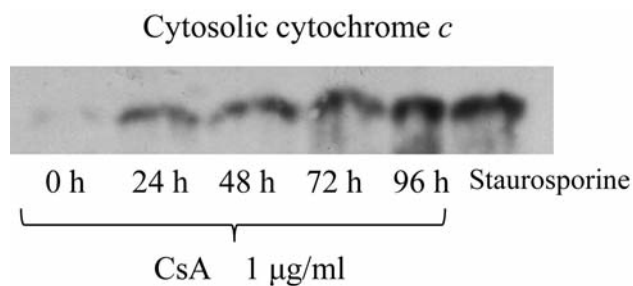


Figure 3. Effect of CsA on cytosolic cytochrome c release in A549 cells. A549 cells were incubated with 1  $\mu$ g/ml CsA for 0, 24, 48, 72 and 96 h. The cytosolic fraction was isolated, and the content of cytochrome c was examined by immunoblot analysis using anti-cytochrome c antibody.

## Discussion

Lung rejection remains a common problem among transplant recipients. Because the side effects of chronic systemic immunosuppression are well known, recent efforts have explored alternative approaches to controlling rejection. Clinical trials have demonstrated that aerosolized CsA can effectively control rejection in lung allografts. Keenan *et al.* found that 9 out of 12 patients (5 with acute and 4 with chronic rejections) exhibited histological resolution of rejection within 3 months of inhaled CsA therapy (17). Iacono *et al.* reported about 39 transplant recipients who received aerosolized CsA therapy in addition to conventional immunosuppression (18). These authors showed that aerosolized CsA therapy in lung transplant recipients with bronchiolitis obliterans provided a survival advantage over conventional therapy alone. Another study showed that survival was improved with aerosolized CsA (3 cases of death among 28 patients receiving CsA and 14 among 30 patients receiving placebo), and that chronic rejection-free survival was also improved with aerosolized CsA (10 and 20 cases in the CsA and placebo groups, respectively) (19). An overview of published reports indicates that local CsA therapy has potential benefits in preventing rejection in lung transplantation.

Mitruka *et al.* compared the lung and blood concentration of aerosolized CsA after its administration into rat lung. CsA concentration in the lung tissues rapidly elevated to 100  $\mu$ g/ml, but then decreased to 10  $\mu$ g/ml within 5 h. The lung concentrations were 10 times higher than that of blood at 24 h (4). The CsA concentrations used in our study (0.5-2.0  $\mu$ g/ml) were 5-20 times higher than those used in a clinical setting with oral administration (20, 21).

We used the clonogenic assay technique, which is an established method to determine the cytotoxic effects of anticancer agents. The results of this assay clearly show that CsA exposure reduced the colony count of lung cancer cells in a concentration-dependent manner. Because there are no reports in the literature concerning the effect of CsA on

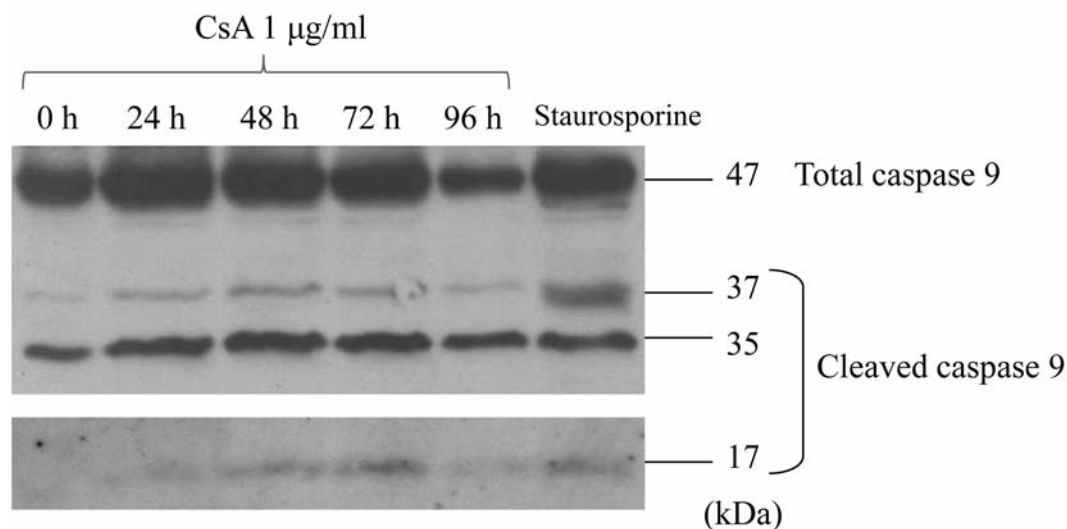


Figure 4. Effect of CsA on caspase-9 activity in A549 cells. A549 cells were treated with either 1 µg/ml CsA for 0, 24, 48, 72 and 96 h, or 2 µM staurosporine for 4 h. Cell extracts were then subjected to Western blotting using anti-caspase-9 antibodies. Staurosporine was used as the positive control.

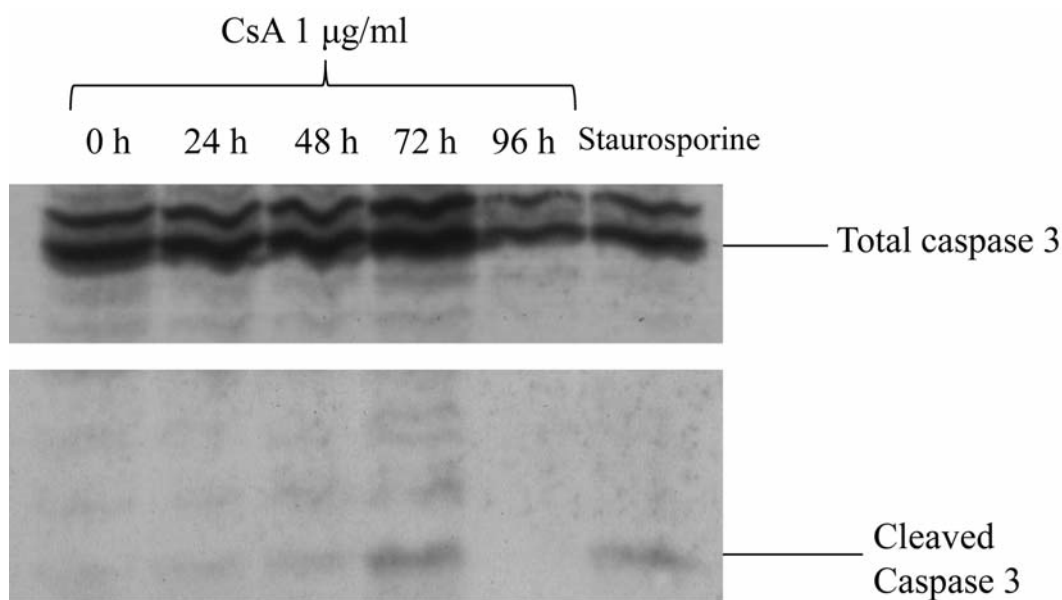


Figure 5. Effect of CsA on caspase-3 activity in A549 cells. A549 cells were treated with either 1 µg/ml CsA for 0, 24, 48, 72 and 96h or 2 µM staurosporine for 4 h. Cell extracts were then subjected to Western blotting using anti-caspase-3 antibodies. Staurosporine was used as positive control.

apoptosis of lung cancer, we next examined whether CsA could induce apoptosis of lung cancer cells. In the present study, the apoptotic cell population was identified immunocytochemically using Annexin V, a marker of cells in the early stage of apoptosis. Annexin V/PI staining showed that 1 µg/ml of CsA gradually increased the number of early apoptotic cells over 96 h of culturing.

To gain further insights into the molecular events associated with CsA-induced apoptosis, the expression of

apoptosis-related proteins was detected by immunoblotting.

Recent studies suggest that at least two different pathways lead to activation of caspases and induction of apoptosis, both pathways being distinguished by the accumulation of cytosolic cytochrome *c* (22, 23). The mechanism of cytochrome *c*-dependent apoptosis is well known (24, 25). Caspase-3 is believed to be activated by an Apaf complex consisting of Apaf-1 (human homolog of CED-4 protein), Apaf-2 (cytochrome *c*), and Apaf-3 (procaspase-9) (26, 27).



Cytochrome *c* released from the mitochondria during apoptosis induction plays a key role in the recruitment of caspase-9 to Apaf-1, leading to activation of caspase-9 and eventual processing of procaspase-3 (28). The results of immunoblot analysis in this study showed that CsA therapy resulted in cytochrome *c* release from the mitochondria to the cytoplasm, and activation of caspase-3.

Caspase 8 is probably activated in most apoptotic systems, including cell death induced by various chemotherapeutic drugs, such as etoposide, chlorambucil, fludarabine, cisplatin, doxorubicin and 5-fluorouracil. Some drugs may rapidly up-regulate death receptors (*e.g.* DR5 and FAS) or their ligands (*e.g.* FAS ligand, TRAIL and TNF- $\alpha$ ), thereby directly activating the death receptor-mediated activation of caspase-8 (29-34). However, our data showed that CsA did not activate caspase-8 in A549 lung cancer cells. Caspase-9 appears to be a functionally important initiator of the apoptotic cascade. In contrast to other caspases, the unprocessed form of caspase-9 is enzymatically active, and by processing itself its activity increases by a factor of approximately 10. Pro-caspase-9 and processed caspase-9 are active only in the presence of their co-activators (Apaf-1/cytochrome *c* complex). Invalidation of the caspase-9 gene and transfection with dominant-negative mutants of caspase-9 have revealed its critical role in developmental cell death (particularly in neurons), as well as in chemotherapy responses *in vitro* (35-37). In particular, in the mitochondrial pathway of cell death induction, caspase-9 appears to be necessary for the occurrence of full-blown apoptosis (38, 39). In the present study, we showed that the level of cleaved caspase-9 was dramatically increased by CsA therapy in a dose-dependent manner. Therefore, our data suggest that the mitochondria and caspase-9 activation pathway is critical in CsA-induced lung cancer cell apoptosis.

We have thus demonstrated that CsA-induced apoptosis of lung cancer cells occurs through the mitochondrial and caspase-9 activation pathway. In contrast to lung cancer cells, normal structural lung cells are highly protected from CsA-induced apoptosis, considering that there are only few reports about pulmonary toxicity from inhalation therapy. This characteristic feature of CsA supports its safety and suitability for lung transplantation patients. In addition, it also suggests that local administration of CsA will provide a regional advantage in the prevention of lung rejection without secondary lung cancer.

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