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α-Melanocyte Stimulating Hormone (MSH) Decreases Cyclosporine A Induced Apoptosis in Cultured Human Proximal Tubular Cells

The pathogenesis of chronic cyclosporine A (CsA) nephrotoxicity has not been elucidated, but apoptosis is thought to play an important role in CsA induced tubular atrophy. Recently Fas-Fas ligand system mediated apoptosis has been frequently reported in many epithelial cells as well as in T lymphocytes. We investigated the ability of CsA to induce apoptosis in cultured human proximal tubular epithelial cells and also the effect of α-MSH on them. Fas, Fas ligand, and an intracellular adaptor protein, Fas-associating protein with death domain (FADD) expression, and poly-ADP ribose polymerase (PARP) cleavage were also studied. CsA induced apoptosis in cultured tubular epithelial cells demonstrated by increased number of TUNEL positive cells and it was accompanied by a significant increase in Fas mRNA and Fas ligand protein expressions. FADD and the cleavage product of PARP also increased, indicating the activation of caspase. In α-MSH co-treated cells, apoptosis markedly decreased with downregulation of Fas, Fas ligand and FADD expressions and also the cleavage product of PARP. In conclusion, these data suggest that tubular cell apoptosis mediated by Fas system may play a role in tubular atrophy in chronic CsA nephrotoxicity and pretreatment of α-MSH may have a some inhibitory effect on CsA induced tubular cell apoptosis.

Key Words : *α-MSH; Apoptosis; Cyclosporine Nephrotoxicity; Fas System*

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INTRODUCTION

The introduction of cyclosporine A (CsA) in organ transplantation greatly improved graft survival but the clinical utility of CsA has been limited by the frequent occurrence of chronic nephrotoxicity, characterized by striped interstitial fibrosis, tubular atrophy and progressive renal functional impairment (1, 2). Because CsA is known to induce intense afferent arteriolar vasoconstriction (3, 4), it has been presumed that the above tubulointerstitial changes are the result of chronic low grade ischemia. However recent studies showed that CsA could be directly toxic to renal tubular cells with site selective action on proximal tubule (5, 6).

Apoptosis, a distinct form of cell death, can serve as a molecular selection process for normal development and various disease processes (7-9) and has also been reported to occur in chronic CsA nephrotoxicity animal model as well as in cultured tubular cell upon CsA stimulation (10-12). It suggests that apoptosis can be another possible pathogenetic mechanism of chronic CsA nephrotoxicity, but the mechanism of apoptosis in CsA nephrotoxicity remains poorly defined.

Fas (APO-1/CD95)/Fas ligand (FasL) system is the best characterized system that can mediate apoptotic cell death in many cell types and also expressed in renal tubular cells (13-16).

The initiation of downstream signaling events in response to Fas/FasL interaction requires a complex network of intermediate signaling proteins interacting with the cytoplasmic domain of receptors via the conserved death domain (17). One of them is Fas-associating protein with death domain (FADD) or MORT1 and the subsequent results of the signaling process is the activation of caspase 8 (FLICE/MACH), followed by the activation of caspase 3 (CPP32/YAMA/APOPAIN) with cleavage of several substrates like poly-ADP ribose polymerase (PARP), lamin, and actin (17, 18).

Schelling et al. recently observed that upregulated tubular cell Fas is responsible for tubular cell loss in chronic renal failure. This finding suggests that Fas system-mediated tubular cell apoptosis can also be the principal mechanism of apoptosis in tubular atrophy in chronic CsA nephrotoxicity (19).

α-melanocyte stimulating hormone (α-MSH) is a proopiomelanocortin derivatives and is an endogenous cytokine that suppresses the inflammation in various animal models by way of its inhibitory action on proinflammatory cytokines and chemoattractant chemokines (20-23). Recently, Chiao

et al. reported a beneficial effect of α-MSH in murine model of ischemic acute renal failure (ARF) through an inhibition of mouse chemokine KC and intercellular adhesion molecule-1 (ICAM-1) expression that mediates the adherence of neutrophils to endothelium and the subsequent neutrophil infiltration (24). In addition, we have observed that tubular cell apoptosis also significantly decreased in α-MSH treated group in ischemic ARF rat model with concomitant decrease in Fas/FasL protein expression and suggested that the beneficial effects of α-MSH might be partially related to its inhibitory action on Fas/FasL system and apoptosis (25).

The aim of this study was to examine the capacity of CsA to induce apoptosis in cultured human proximal tubular cells and also Fas/FasL expression with downstream signaling molecule, FADD and PARP cleavage as a possible mechanism of CsA induced apoptosis as well as the effect of α-MSH on them.

MATERIALS AND METHODS

Cell culture and experimental conditions

HK-2 (human kidney-2) cells, an immortalized proximal tubular epithelial cell line from normal adult human kidney were obtained from American Tissue Culture Collection (Rockville, MD, U.S.A.). Cells were grown in DMEM/F12 medium (Dulbeco's modified Eagle's medium: Nutrient Mixture F12 (Ham) 1:1 ratio), GIBCO BRL (Life Technology, Grand Island, NY, U.S.A.) supplemented with 5% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 5 μg/mL transferrin (GIBCO BRL), 5 ng/mL sodium selenite (Sigma Chemical Co., St. Louis, MO, U.S.A.), 5 pg/mL T3, 5 ng/mL hydrocortisone, 5 pg/mL PGE1, and 10 ng/mL epidermal growth factor (Sigma). For passage, confluent cells were washed with PBS, and removed with Trypsin-EDTA (GIBCO BRL), and plated in DMEM/F12 complete media. Cells in the 4th through 6th passage were used. After incubating the cells in serum and growth factor-free basal medium for 24 hr, CsA (Novartis Pharmaceutical Ltd) in various concentrations (500, 1,000, and 10,000 ng/mL) were added and incubated for 24 hr. α-MSH (1 μM) was added 1 hr prior to addition of CsA (1,000 ng/mL). Identical volume of olive oil was used as vehicle (V) and normal control cells (NC) were incubated with medium alone. Two independent experiments were performed in triplicate.

Reverse transcription-PCR

Total RNA was extracted from the cells by TRIZOL (GIBCO BRL) according to the manufacturer's protocol. RNA concentrations were determined using spectrophotometric readings at A_{260}. One microgram of RNA was reverse transcribed at 42℃ for 60 min in a 25 μL reaction mixture with 5× first strand buffer, 10 mM dNTP, 20 U RNAsin, and 500 U of Moloney murine leukemia virus reverse transcriptase (Superscripts, GIBCO BRL, Grand Island, NY, U.S.A.). First strand cDNA (1 μL) was amplified using 2.5 U *Taq* polymerase (Perkin Elmer, Foster city, CA, U.S.A.) in a 50 μL reaction volume containing 0.4 μM primer pair, 200 μM dNTP, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, and 50 mM KCl. The sequences of primer for human Fas were: sense, 5′-TCTGTT CTGCTGTGTCTTGG-3′; antisense, 5′-GGG TGGCTTT GTCTTCTTCT-3′ and human ribosomal protein L-19 as an internal standerd were: sense, 5′-AGCCTGTGACTGT CCATTCC-3′ antisense, 5′-TTG-GTCTTAGACCTGCG AGC-3′. The amplifying conditions were repeated for 36 cycles of the following: denaturation for 60 sec at 94℃, annealing for 60 sec at 58℃, and extension for 60 sec at 72℃. The PCR products were analyzed on a 1% agarose gel and stained with ethidium bromide. The band densities were determined using a Digital Imaging & Analysis System (Alpha Innotech Corp., San Leandro, CA, U.S.A.). The ratios of Fas to L-19 PCR products were expressed as percent of control mean in each groups and compared between normal control, CsA, and CsA+α-MSH group.

Western blot analysis

Both adherent and nonadherent cells were washed with PBS and then solubilized in 2× SDS sample buffer, and then boiled for 10 min. The samples were initially analyzed by 10% SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue to assure identical loading (Bio-Rad Mini Protean II). Aliquots of each sample containing the same amount of protein were resolved in a 10% SDS-polyacrylamide gel and transferred to polyvinylidine difluoride (PVDF) membrane. After incubation in a blocking solution [5% nonfat dry milk in PBST (0.05% Tween 20 in PBS)] at room temperature for 1 hr, the membranes were washed repeatedly with PBST (4× 10 min), and then incubated with primary antibodies in a blocking solution at 4℃ overnight. After the washing, the membranes were reacted with a secondary antibody conjugated with horseradish peroxidase (anti-mouse IgG HRP, Santa Cruz Biotechnology, CA, U.S.A.) at room temperature for 1 hr. The primary antibodies used were: (1) 1:2,000 mouse anti-FasL IgG (Transduction Laboratories, Lexington, KY, U.S.A.), (2) 1:500 mouse anti-FADD IgG (Transduction Laboratories), (3) 1:10,000 mouse anti-PARP monoclonal antibody (CLONTECH Laboratories, Palo Alto, CA, U.S.A.). After washing in PBST (8× 10 min), the membranes were visualized using enhanced chemiluminescence (ECL: Amersham, Arlington Heights, IL, U.S.A.) and exposed to Kodak XAR5 film. The band intensities were expressed as a percent of control mean and compared between the normal control, V, CsA, and CsA+α-

MSH groups using the Digital Imaging & Analysis System (Alpha Innotech Corp., San Leandro, CA, U.S.A.).

Detection of apoptosis

DNA fragmentation was examined by using the TUNEL method (ApoAlert® DNA Fragmentation Assay Kit, CLONTECH Laboratories, Palo Alto, CA, U.S.A.). Adherent cells, grown in coverslide in six well plate under the experimental conditions as above, were washed with PBS and fixed with 4% formaldehyde/PBS for 30 min at 4℃. After the cell membrane were permeabilized with 0.2% Triton X-100/PBS for 15 min at 4℃, cells were incubated with a mixture of nucleotide and TdT enzyme for 60 min in a dark, humidified incubator at 37℃. The reaction was terminated with 2×SSC and washed with PBS. After the mounting of glass slide, fluorescent nuclei were detected by fluorescein filters (BH2: Olympus). TUNEL positive cells were counted in 5×200 filelds and mean number was compared between groups.

Statistical analysis

Results are presented as mean percentage±SEM of normal control value from two independent experiments, each performed in triplicate. Comparisons between the normal control and CsA stimulated groups, CsA and CsA+α-MSH groups were done using independent sample t-test and a *p*-value less than 0.05 was considered to be statistically significant.

RESULTS

CsA induced Fas mRNA expression in cultured human proximal tubular cells

Fas/L-19 ratio increased from 100±7.2% in normal control group to 132.5±18.8%, 146.3±0.7%, 116.3±13.7% in CsA 500, 1,000, 10,000 ng/mL treated groups, respectively but this trend was not statistically significant except CsA 1,000 ng/mL group (p=0.15, p<0.01, and p=0.32 respectively). In α-MSH co-treated group, Fas/L-19 ratio decreased to 60±8.7% of normal control and it was a statistically significant decrease compared to CsA 1,000 ng/mL group (p<0.01) (Fig. 1).

CsA induced Fas ligand protein expression in cultured human proximal tubular cells

FasL protein was basally expressed in a low level in cultured proximal tubular cells (100±10.1%) and the expression significantly increased by CsA stimulation (CsA 500,

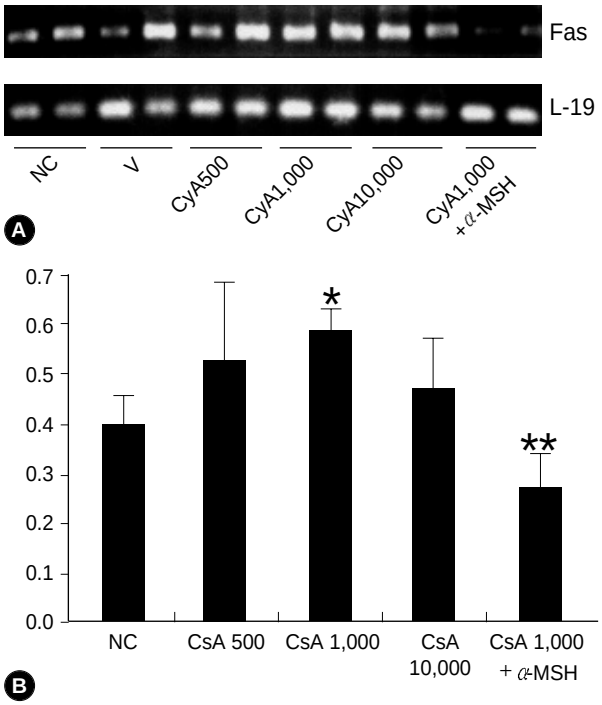

Fig. 1. Semiquantitative RT-PCR for human Fas. Compared with normal control group, CsA treatment upregulated Fas mRNA expression in cultured human proximal tubular cells. In CsA (1,000 ng/mL)+α-MSH treated group, Fas mRNA expression significantly reduced. *p<0.05 compared with normal control group, **p<0.05 compared with CsA 1,000 ng/mL group.

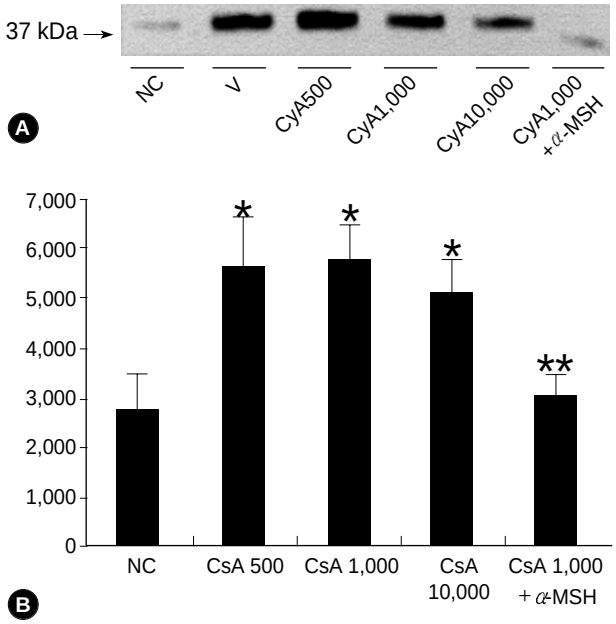

Fig. 2. Western blot for Fas ligand. CsA increased Fas ligand protein expression in cultured human proximal tubular cells and in CsA+α-MSH group, expression of Fas ligand protein decreased significantly. *p<0.05 compared with normal control group, **p<0.05 compared with CsA 1,000 ng/mL group.

CsA 1,000, CsA 10,000 ng/mL: 204.4±21.5, 208.7±15.1, 184.7±14.2%; $p<0.01$, $p<0.01$, and $p<0.01$, respectively). In α-MSH co-treated group, the expression decreased to 110.4±12.2% compared to CsA 1,000 ng/mL group ($p<0.01$) (Fig. 2).

CsA induced FADD protein expression in cultured human proximal tubular cells

FADD was also basally expressed in cultured proximal tubular cell and upon CsA stimulation, the expression increased significantly (CsA 500, CsA 1,000, CsA 10,000 ng/mL: 190.0±18.8, 186.8±7.5, 159.0±2.2%: $p<0.01$, $p<0.01$, and $p<0.01$, respectively). In α-MSH co-treated group, FADD expression decreased to 121.9±1.7% of that of normal control compared to CsA 1,000 ng/mL treated group ($p<0.01$) (Fig. 3).

CsA increased the cleavage product of PARP indicating the activation of caspase

Because Fas/FasL mediated apoptotic pathway results in the activation of caspase with cleavage of many substrates like PARP, lamin, and fodrin, we examined a cleavage of PARP, a DNA repair enzyme that is mainly cleaved by caspase 3. In a basal condition, PARP exists predominantly as a 116 kDa, full-length peptide, but in CsA stimulated condition, 89 kDa cleavage product increased indicating the activation of caspase 3. In α-MSH co-treated group, the cleavage product decreased compared to CsA treated group. We used TNF-α, a well known proapoptotic cytokine, as a positive control (Fig. 4).

CsA increased apoptosis in cultured human proximal tubular cells

Compared to normal control vehicle treated cells, the number of TUNEL positive cells increased in CsA treated cells, but decreased in α-MSH co-treated cells (Fig. 5). Mean number of TUNEL positive cells per ×200 field in CsA treated cells signiticantly increased compared with in normal control cells ($p<0.05$), and decreased significantly in α-MSH co-treated group ($p<0.05$, vs CsA treated group) (normal control/CsA 1,000/mL/α-MSH CsA 1,000+α-MSH: 4.6±0.6/15.7±0.9/7.2±0.6).

DISCUSSION

In the present study, we have demonstrated that CsA induced apoptosis in cultured human proximal tubular cells. The CsA concentrations (500-1,000 ng/mL) used in this study were to represent the approximate concentration range in in vivo kidney tissue, and thus a nephrotoxic in vitro model simulating clinical setting (26). Apoptosis, a genetically regulated form of cell death has been observed in various renal diseases, e.g., ischemia-reperfusion injury, transplant rejection (27, 28) as well as other conditions characterized by progressive tubulointerstitial injury and glomerulosclerosis (19). In chronic CsA nephrotoxicity model, increased apoptosis of tubulointerstitial cells were also observed and it correlated with pathologic findings such as tubular atrophy and interstitial fibrosis (10, 11). The mechanism for this increase in apoptosis is not clear, but considering the known vasoconstrictive effect of CsA, chronic low grade ischemia is thought to play a role. This observation is in line with the

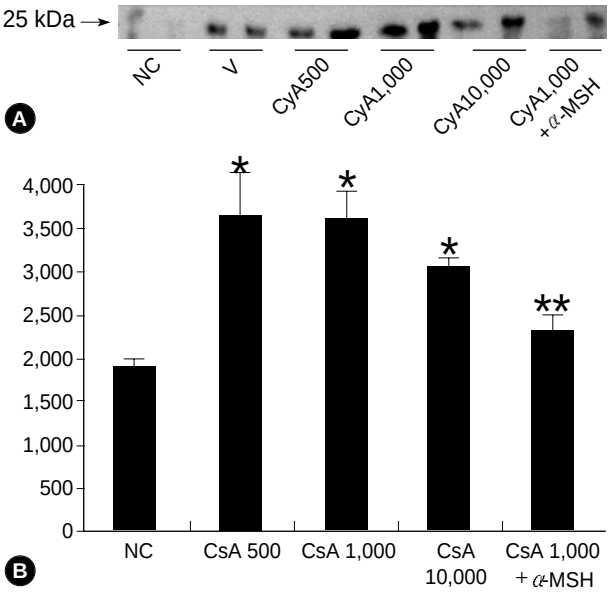

Fig. 3. Western blot for FADD. Compared to normal control group, FADD expression increased significantly in CsA treated groups. In CsA+α-MSH group, FADD expression decreased. *$p<0.05$ compared with normal control group, **$p<0.05$ compared with CsA 1,000 ng/mL group.

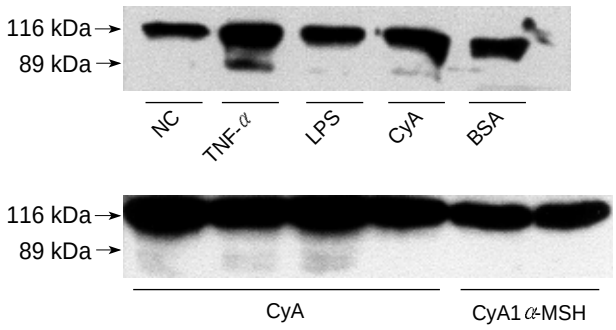

Fig. 4. Western blot for poly-ADP ribose polymerase (PARP). The 89 kDa cleavage product increased in CsA group compared with normal control group, indicating the activation of caspase. In α-MSH treated group, the cleavage product decreased. A proapoptotic cytokine TNF-α was used as a positive control.

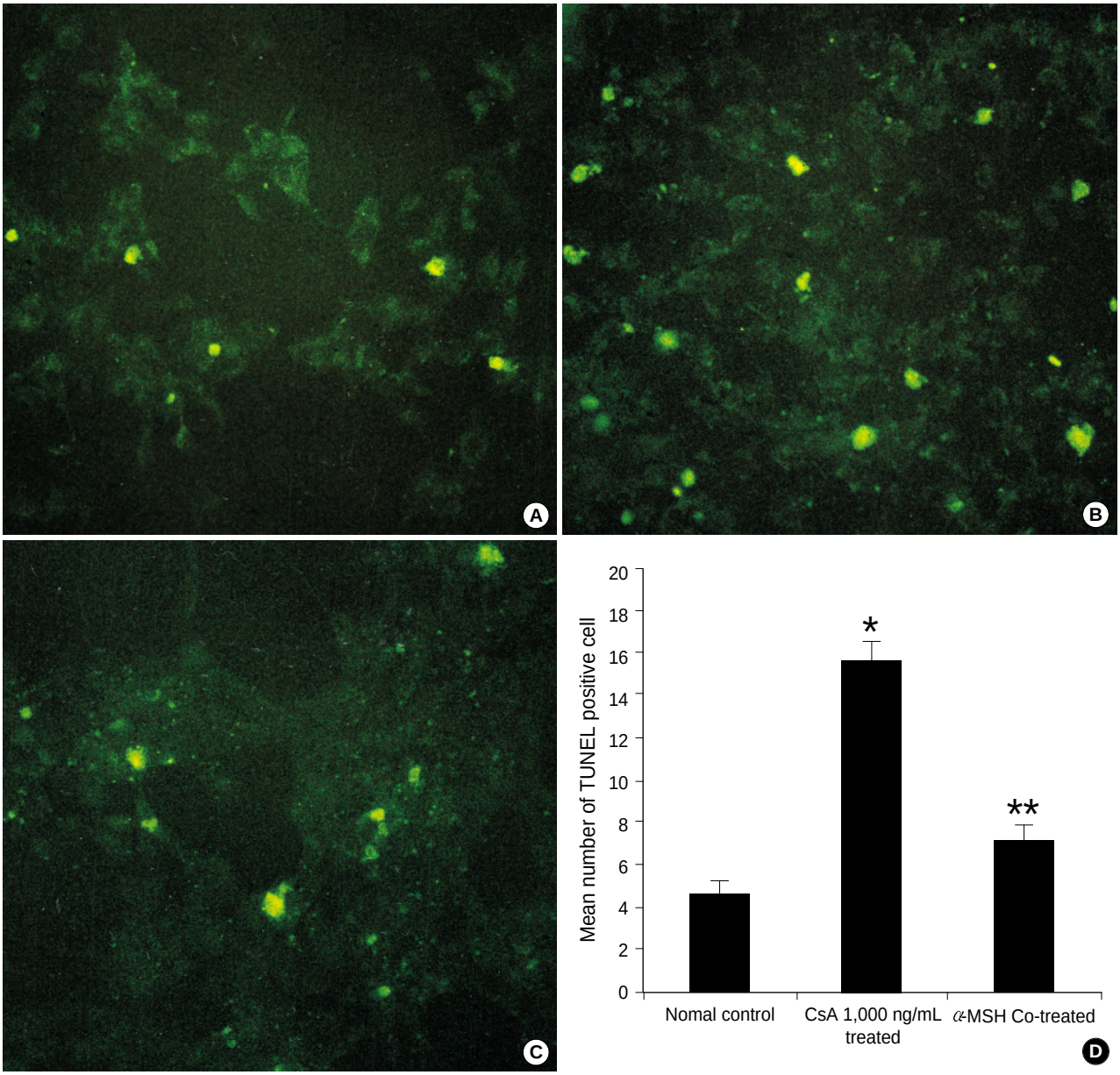

Fig. 5. In situ end labelling of apoptosis (×200). Compared with normal control group in which there were few TUNEL positive apoptotic cell, apoptosis increased in CsA treated group. In CsA+α-MSH treated group, apoptosis decreased markedly suggesting its inhibitory effect on tubular cell apoptosis. (A) normal control, (B) CsA 1,000 ng/mL, (C) CsA 1,000 ng/mL+α-MSH (1 μM). (D) mean number of TUNEL positive cells in each group. Compared with normal control, TUNEL positive cells increased markedly in CsA 1,000 ng/mL treated group (*p<0.05), and TUNEL positive cells decreased significantly in α-MSH co-treated group compared with CsA 1,000 ng/mL treated group (**p<0.05 vs CsA 1,000 ng/mL treated group).

data that apoptosis was augmented by angiotensin II (Ang II) and decreased by nitric oxide (NO) (10), but using an in vitro culture system, direct toxicity to tubular cells has also been documented (12, 29). In this study, we could demonstrate that apoptosis as assessed by DNA fragmentation apparently increased in CsA treated cells. The capacity of CsA to induce apoptosis in tubular cells may be clearly related to tubular atrophy in chronic CsA nephrotoxicity, because even very small increase in apoptosis can result in a very significant loss of cells in vivo due to the relatively short half life of apoptotic cells.

Although it is apparent that tubular cell apoptosis induced by CsA contribute to its nephrotoxicity, the molecular pathways that lead to apoptosis is not clear. Fas/Apo-1/CD95, a 45 kDa transmembrane protein is the best characterized receptor superfamily that can trigger apoptosis in various cells

and also expressed in renal tubular epithelial cells (14-16). The ligand for Fas belongs to tumor necrosis factor (TNF) superfamily and is also known to be expressed in renal tubular cells (13). Recently, Fas/FasL system mediated apoptosis is reported to play an important role in endotoxemia induced ARF and tubular atrophy in animal models of chronic renal failure (19, 30). The possbile involvement of Fas system in chronic CsA nephrotoxicity has also been suggested by Healy et al. who demonstrated the increased Fas protein expression in a CsA stimulated condition (12). Ortiz et al. also suggested a role for the autocrine activation of Fas system in CsA nephrotoxicity by observing that CsA induced apoptosis decreased by inhibitor of caspase 8, an upstream effector of death receptor mediated apoptosis (29). In this study, we also observed the increase of Fas mRNA expression in CsA group compared with that in control or vehicle group. FasL and subsequently FADD, an intracellular adaptor protein that binds to a conserved amino acid sequence known as "death domain" on the cytoplasmic domain of Fas receptor, also increased in CsA group. Interestingly, in high dose CsA treated group (10,000 ng/mL), further increase in Fas, FasL, and FADD expressions was not observed any more. This result raises a possibility that in the setting of high dose CsA, cell necrosis predominates rather than Fas system mediated apoptosis. Healy et al. had also observed that in high dose CsA group, membrane integrity decreased significantly together with increased LDH release indicating cell necrosis rather than apoptosis (12).

We also examined the cleavage of PARP, a nuclear protein involved in DNA repair, mainly cleaved by caspase 3. Caspase 3 (CPP32/Yama/Apopain) is regarded as one of the central executioner molecule in apoptotic pathway and in CsA treated group, we observed the increase in 89 kDa PARP cleavage product indicating the activation of caspase. Previously, the role of caspase in CsA induced apoptosis has been suggested by Ortiz et al. who demonstrated that caspase inhibitor significantly decreased apoptosis (29).

In addition, we were interested in the ability of α-MSH in decreasing Fas/FasL mediated apoptosis in tubular cells because we had previously observed that tubular cell apoptosis significantly decreased in α-MSH treated group with a concomitant decrease in Fas/FasL expression in ischemic ARF rat model (25). α-MSH, an endogenous antiinflammatory cytokine, has been known to reduce cellular infiltrations in various inflammatory conditions such as rat model of arthritis and liver injury from septic shock (20-23). α-MSH also has been reported to have a beneficial effect in ischemia/reperfusion injury through its inhibitory action on the mouse chemokine KC and ICAM-1 messages and on the induction of iNOS with a resultant decrease in peroxynitrate production (24). In our previous study, intraperitoneally administered α-MSH was also effective in attenuating the severity of injury determined by biochemical and histological data. In addition, α-MSH also decreased tubular cell apoptosis at 24 hr after reperfusion when the severity of injury peaked with concomitant decrease in Fas/FasL expression (25). However, an increase of Fas/FasL expression can be secondary to inflammatory cytokines produced by infiltrating neutrophils or tubular cells, and downregulation of the expression in α-MSH treated group could also be secondary epiphenomenon due to decreased cytokine production. So with in vivo study, it was difficult to distinguish between these two possibilities.

But in the present study, using an in vitro culture system, α-MSH, administered 1 hr prior to the addition of CsA, significantly reduced Fas/FasL and FADD protein expressions as well as the cleavage of PARP, which was pararelled by reduced DNA fragmentation. These results indicate that α-MSH has a direct inhibitory effect on death receptor mediated apoptosis in tubular cells. The effect of α-MSH on apoptosis or Fas/Fas ligand expression has not been studied previously. And the possible role of α-MSH in inhibition of Fas/FasL expression and apoptosis and the precise mechanism thereof need further studies.

In conclusion, our data suggest that CsA induced tubular cell apoptosis may play an important role in tubular cell loss in chronic CsA nephrotoxicity and Fas/FasL system can be an important mediator of CsA induced apoptosis. α-MSH, a nonspecific antiinflammatory cytokine can be a potential therapeutic agent for CsA nephrotoxicity through its inhibitory effect on Fas/FasL expression and apoptosis.

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