Mechanisms of Effect of Arjunolic Acid on Cyclosporine A Induced Renal Tubular Cell Apoptosis

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Abstract—Apoptosis is an important pathogenic mechanism in renal diseases, during development and injury. Lethally injured cells may die by either necrosis or apoptosis, depending on the severity of the injury. Severe renal ischemia results in acute tubular necrosis, chronic low grade ischemia leads to apoptosis in renal tubular cells. Cyclosporine-induced apoptotic cell death is closely associated with activation of proapoptotic genes and altered regulation of apoptosis-regulating genes. Cyclosporine A (CsA) has considerably modified the graft survival in solid organ and bone marrow transplantation. Renal toxicity is the major adverse effect of chronic CsA administration. Deterioration of renal function and renal histopathology are the basic elements of the diagnosis. In the present study, the lethal pathways activated by cyclosporine A (CsA), a nephrotoxin that induces caspase-dependent apoptosis in tubular epithelium, were explored. The Tubular cell apoptosis is the major contributing factor to the pathogenesis of renal injury. The understanding of the CsA renal effects is not only a research interest, but also clinically important in developing a strategy in the prevention and treatment of the disease. In this contribution, we will discuss this effect and its significance for the mechanism of action of Bax, cytochrome c, caspase-3 in renal tubular cells, an important checkpoint of the apoptotic process.

Key words: Cyclosporine, Apoptosis, Arjunolic acid

I. INTRODUCTION

Apoptosis is a programmed cell death, possesses characteristic morphological and biochemical changes (1-3). Apoptosis plays important role in regulating renal cell number in health and disease (4,5). Apoptosis is the mechanism of cell clearance and progression of kidney injury in several animal models, including ischemia-reperfusion injury (6). Chronic kidney disease, (7) diabetic nephropathy, (8) and obstructive kidney disease (9). The major multigene families involved in the molecular controls of cell survival or death is the Bcl-2 gene family. Its members include inhibitors (eg, Bcl-2, Bcl-XL, Bcl-w, and Mcl-1) and accelerators of apoptosis (eg, Bax, Bcl-Xs, Bak, Bik, and Bad) (10). Heterodimerization between anti apoptotic Bcl-2 and pro apoptotic Bax may negate the function of either protein. The ratio of Bcl-2 to Bax appears to determine a cell’s fate (11,12).

Caspases are the family of cysteine proteases and are activated during programmed cell death. Among them, caspase-3 (CPP32/Yama/apopain) is an executor of apoptosis (10). Several recent studies have demonstrated that cyclosporine treatment directly induces apoptosis in the renal tubular cell line and human proximal tubular epithelial and endothelial cells in vitro (13-15). Cyclosporine-induced apoptotic cell death is closely associated with activation of proapoptotic genes and altered regulation of apoptosis-regulating genes (16).

Apoptosis has been clearly evidenced in tubular and interstitial cells of transplanted patients with chronic cyclosporine nephrotoxicity (17). Tubular cell apoptosis is also observed in animal (18-20) and cell culture model (21, 22). Cyclosporine-induced apoptosis is primarily triggered through the mitochondrial pathway. The generation of ROS (indirectly demonstrated in vitro in tubular epithelial cells through the protective effect of prednisone (23), the reduction of Bcl-2 and IAP expression, the increased expression of Bax (in mesangial cells (24) and well as in vivo (20) and the translocation of Bax to the mitochondria (in murine tubular epithelial cells (25) all contribute to apoptosis induction.

The use of medicinal plant either as a single drug or in combination is increasing in the health care of human being. Medicinal plants can be important source of previously unknown chemical substances with potential therapeutic effect. Terminalia arjuna bark is commonly known as arjuna bark or arjun and abundantly available throughout the India. Terminalia arjuna possesses wide-ranging therapeutic properties and has the potential to treat numerous medical conditions, especially those pertaining to the heart and circulation system. It is considered to be an excellent hypolipidemic, anticoagulant, hypocholesteremic, antihypertensive, antiviral, antithrombotic, antifungal and antibacterial properties (26). In this review, we showed that Bax, cytochrome c, caspase-3, induce changes observed in the kidney of cyclosporine-induced renal cell apoptosis, and discussed the protective effect of arjunolic acid on the art of apoptosis modulation in nephrotoxic kidney injury.

II. MATERIALS AND METHODS

A. Extraction of Arjunolic Acid

The extraction of Arjunolic acid was carried out by following the methods of King et al., (1954) (26) and Bag et al., (2005) (27). Briefly, after collection the bark of Terminalia Arjuna was cut into small pieces, dried and ground into powder (1 kg) which was then extracted with petroleum ether (20 h) to remove greasy non polar material. After removing the petroleum ether the crude material was subsequently extracted with diethyl ether (40 h). The crude material was filtered off and the mother liquor was concentrated under reduced pressure. Next the compound was subjected to separation on silica gel column. Elution with chloroform: methanol and the arjunolic acid were obtained in the fractions were pooled & tested the identity of the presence of AA by TLC and colour reaction with anisaldehyde. The extract was lyophilized. The purity of the compound [Arjunolic acid] has been checked by using standard tool like NMR and IR. The extract containing Arjunolic acid is indicated as Arjunolic acid (AA).
B. Animal Model

Male albino rats of wistar strain weighing (180±20g) were obtained from the laboratory animal maintenance unit, Saveetha University, vellapanchavadi, Chennai. The animals were acclimatized to the laboratory conditions for a period of one week. They were maintained at an ambient temperature of 25±2°C and were given standard rat feed and water ad libitum. The experiments were conducted according to the ethical norms approved by ministry of social justice and empowerment, Government of India and institutional Animal Ethics Committee guidelines (IAEC No. Biochem BWC.006 2009).

III. EXPERIMENTAL DESIGN

The following groups of animals will be used for the present study. The rats will be divided into four groups (n=6) in each group.

- GROUP I: Control treated with vehicle alone.
- GROUP II: Cyclosporine A (CsA) induced orally (25 mg/kg body wt.) for a period of 21 days.
- GROUP III: Cyclosporine A induced (25 mg/kg body wt.) + Arjunolic acid (10mg /kg body wt.) treated orally.
- GROUP IV: Arjunolic acid treated orally (10mg /kg body weight) for a period of 21 days.

IV. PREPARATION OF KIDNEY HOMOGENATE

Within 3 hours after sacrifice, kidney samples were blotted to dryness. From this, a piece weighing about 100mg was taken and homogenized at 4°C in Tris-HCl buffer (0.1M, pH 7.4). The tissue homogenate was centrifuged at 2,500 rpm for 30 minutes. The resultant supernatant was kept under refrigeration until further biochemical analysis. All the assay procedures were carried out within 48 hrs after sample collection.

V. WESTERN BLOTTING

For immunoblot analysis, kidney tissue was homogenized in 4°C in Tris-HCl buffer (0.1 M, PH 7.4). The tissue homogenate was centrifuged at 2500 rpm for 30 minutes and the protein concentration was determined by lowry’s method (31). Protein samples were resolved on 12% SDS-polyacrylamide gel electrophoresis (SDS – PAGE) and then electroblotted on Bio-Blot nitrocellulose membrane (Bio-RAD, Hercules, CA, USA). The membrane was blocked for 1 hr in TBS added tween-20(TBS-T, Tris-HCL 10mmol/L, Nacl (PH8.0) 150mm/mmol/L, 0.05% tween-20) containing 5% non-fat powered milk. The membrane was incubated with a monoclonal antibody directed against rat Bax, diluted 1:1000. Primary antibody incubation was followed by six washes of TBS-T. The blot was then incubated with secondary antibody (Goat anti rabbit IgG-HRP conjugate at 1:1000 (cell signalling) for 1 hr. The membrane was alternatively washed 3 times for 10 minutes each with TBS-Tween and TBS. The intensity of the immunoreactive bands was monitored by treating the membranes with luminal reagent (Enhanced Chemiluminescence method) according to the manufacturer’s instructions (Millipore, USA). The ECL signals were recorded using a ChemDoc XRS imaging system (Bio-Rad laboratories, Hercules, California, USA) and analyzed using Bio-Rad Quantity One Chem Doc software. The optical densities obtained as 100% reference and normalized with β-actin. The procedure of immunoblotting for (cytochrome -c; Caspase-3, cell signaling with a dilution of 1:1000 and secondary antibody of goat anti rabbit IgG-HRP conjugate, 1:1000) was similar to that of Bax.

VI. DATA ANALYSIS

All the values are represented as mean ± S.D. (n=6). The statistical differences among different groups were analyzed by student’s t-test. P-Values of 0.05 or less were considered significant.

VII. RESULTS

Fig. 1: Western blot expression of Bax in control and experimental groups of animals.

Lane 1, Lane 2, Lane 3 and Lane 4 corresponds to control,CsA,CsA + AA and AA respectively. Quantitative data expressing the corresponding protein levels was assessed using Quantity One ChemDoc software (Bio-Rad) and analyzed using the statistical methods. The statistical differences among different groups were analyzed by student’s t-test. P-Values of 0.05 or less were considered significant.

Fig. 2: Western blot expression of Caspase-3 in control and experimental groups of animals.
Lane 1, Lane 2, Lane 3 and Lane 4 corresponds to control, CsA, CsA + AA and AA respectively. Quantitative data expressing the corresponding protein levels was assessed using Bio-Rad Quantity One ChemDoc software and is expressed in relative intensity arbitrary unit. Values are expressed as mean ± S.D. Comparisons: *Control Vs CsA, ** CsA Vs CsA+AA, ns-not significant, p<0.05.

![Fig. 3: Western blot expression of Cytochrome-c in control and experimental groups of animals.](image)

- **Fig. 3:** Western blot expression of Cytochrome-c in control and experimental groups of animals.

Lane 1, Lane 2, Lane 3 and Lane 4 corresponds to control, CsA, CsA + AA and AA respectively. Quantitative data expressing the corresponding protein levels was assessed using Bio-Rad Quantity One ChemDoc software and is expressed in relative intensity arbitrary unit. Values are expressed as mean ± S.D. Comparisons: *Control Vs CsA, ** CsA Vs CsA+AA, ns-not significant, p<0.05.

Figure (a), shows the western blot analysis of Bax in control and experimental group of animals. In CsA induced (Group-II) rats, increased (Lane 4), expression of Bax was observed. Treatment with AA (Group 3) showed significant reduction (Lane 3) in the expression of Bax comparable with control (Lane 1) and Arjunolic acid alone treated groups (Lane 2).

Figure (b), shows the western blot analysis of Caspase-3 in control and experimental group of animals. In CsA induced (Group-II) rats, increased (Lane 4), expression of Caspase-3 was observed. Treatment with AA (Group 3) showed significant reduction (Lane 3) in the expression of Caspase-3 comparable with control (Lane 1) and Arjunolic acid alone treated groups (Lane 2).

Figure (c), shows the western blot analysis of Cytochrome-c in control and experimental group of animals. In CsA induced (Group-II) rats, increased (Lane 4), expression of Cytochrome-c was observed. Treatment with AA (Group 3) showed significant reduction (Lane 3) in the expression of Cytochrome-c comparable with control (Lane 1) and Arjunolic acid alone treated groups (Lane 2).

**VIII. DISCUSSION**

Bax is a member of Bcl-2-like family of proteins (12), forms heterodimers with Bcl-2. In cells with both Bcl-2 and bax, the Bcl-2/Bax ratio appears as important in determining the susceptibility of the cell to growth factor deprivation-induced apoptosis. Excessive bax predisposes cells to apoptosis under these conditions, but not in the presence of growth factors. The original report suggested that murine kidney expresses two bax transcripts of 1.5 and 1 kb abundantly. Over expression of Bax, the endogenous antagonist of Bcl-2, result in the development of renal cysts.

Apoptosis induced by Cyclosporine A on tubular epithelial cells is related to the translocation of Bax to mitochondria as Bax antisense oligodeoxynucleotides prevented cyclosporine A induced apoptosis (29). Apoptosis and its modulators (Bax and Bcl-2) are associated with progression of tubular atrophy and renal fibrosis in rats, subjected to extensive renal ablation (6). CsA treatment favors Bax expression, which remains diametrically opposed to Bcl-2, raising the possibility of CsA nephrotoxicity and graft survival is improved by regulators of renal apoptosis. Cyclosporine treatment resulted in increased Bax expression, whereas the protein expression of the survival factor Bcl-2 was down-regulated.

Apoptosis is an essential process in the development and tissue homeostasis of most multicellular organisms; deregulation of apoptosis has been implicated in the pathogenesis of CsA-induced nephropathy (13). Previous studies reported that AA reduces acetaminophen induced JNK (Jun N-terminal kinase pathway) and downstream Bcl2 and Bcl-xL, phosphorylation and prevents mitochondrial permeabilization, loss in mitochondrial membrane potential and cytochrome c release (30).

Caspase is discovered as a cytokine-processing enzyme designated as interleukin-1 β-converting enzyme (ICE). Caspase-1 and caspase-11 have been shown to function mainly in cytokine processing (31-33). Whereas, caspase 2, 3, 6, 7, 8, 9, 10 are involved in the regulation and execution of apoptosis (34-37). The functions of the remaining caspases are unknown.

When cells undergo apoptosis, these caspases become activated through one or two sequential proteolytic events and cleave the single peptide precursor into the large and small fragments and constitutes active enzyme (38). Currently two well characterized caspase-activating cascades regulate apoptosis: one is initiated from the cell surface death receptor and the other is triggered by changes in mitochondrial integrity.

Fewer studies on the regulators of apoptosis in CRF models, shows data on the contribution of the caspases, in experimental cyclosporine A–induced nephrotoxicity (15). Specific inhibitors of caspase 2, 3, 8, or 9 decrease apoptosis and prolong cell survival in tubular cells exposed to CsA (29). Murine tubular epithelial cell line exposed to cyclosporine underwent apoptosis, with evidence of caspase-3 activation; the administration of caspase inhibitors prevented the cells from undergoing cyclosporine-induced apoptosis (21).

Cytochrome c is released from mitochondria in cells, undergoing apoptosis induced by a variety of stimuli, includes DNA damaging agents, kinase inhibitors, and activation of cell surface death receptors (39,40). In CsA-treated tubular epithelial cells, Bax translocation and cytochrome c release were caspase-independent phenomena, placing them upstream of or parallel to caspase activation. Bax promotes the release of cytochrome c to the cytoplasm.
where it contributes to the formation of the apoptosome, which leads to the activation of caspase-9 (41).

CsA could significantly increase the Fas/Fas-L, FADD and PARP, induce mitochondrial dysfunction and oxidative stress and injure the antioxidant defense system. It also caused Bax migration to the mitochondria, released cytochrome c into cytosol, downregulated anti-apoptotic proteins (Bcl-2 and BclXL) and increased the activity of caspase (42–45, 14). They finally activated caspase 9 and 3 which mediate cell apoptosis (46–49). Therefore, CsA induces renal cell apoptosis through the Fas/Fas-L pathway as well as mitochondrial pathway with sufficient evidence.

AA represents potential therapeutic option to protect renal tissue from the detrimental effects of acute acetaminophen induced nephrotoxicity is a caspase dependent process that involves the activation of caspase-9 and caspase-3 in the absence of cytosolic cytochrome c release (40). Previous studies reported that AA protects both intrinsic and extrinsic pathways against cadmium induced hepatotoxicity (50).

IX. CONCLUSION

The intracellular components of the apoptosis cascade have now been largely unraveled, revealing specific cellular factors and pathways that can be used as targets and should enable us to design strategies aiming at controlling cell death responses. CsA nephrotoxicity induce changes in Bax, Caspase-3, Cytochrome-c in experimental models. Reduction of the Bax, Caspase-3, Cytochrome-c expression, may account for the beneficial effect of AA on mitochondrial renal damage induced by CsA.

The present data support the assumption about the beneficial effect of AA on renal damage associated with immunosuppressive drug (CsA). We, however, need still to better understand the cross talks between different pathways, to control the cell specificity of the interventions, and to define optimal therapeutic schemes. While highly challenging, the approaches outlined in this review may allow bringing promising preclinical findings to actual therapeutic practice. Further investigation for exploring the beneficial effect of this drug and its usefulness in human nephrotoxicity is recommended.

REFERENCES


