Original Paper

Determinants of Monocyte Apoptosis in Cardiorenal Syndrome Type 1

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Keywords

Cardiorenal syndrome · Caspase · Apoptosis · Monocytes

Abstract

Background: Cardiorenal syndrome type 1 (CRS type 1) is characterized by a rapid worsening of cardiac function leading to acute kidney injury (AKI). Its pathophysiology is complex and not completely understood. In this study, we examined the role of apoptosis and the caspase pathways involved. **Material and Methods:** We enrolled 40 acute heart failure (AHF) patients, 11 of whom developed AKI characterizing CRS type 1. We exposed the human cell line U937 to plasma from the CRS type 1 and AHF groups and then we evaluated apoptotic activity by annexin-V evaluation, determination of caspase-3, -8 and -9 levels, and BAX, BAD, and FAS gene expression. **Results:** We observed significant upregulation of apoptosis in monocytes exposed to CRS type 1 plasma compared to AHF, with increased levels of caspase-3 ($p < 0.01$), caspase-9 ($p < 0.01$), and caspase-8 ($p < 0.03$) showing activation of both intrinsic and extrinsic pathways. Furthermore, monocytes exposed to CRS type 1 plasma had increased gene expression of BAX and BAD (intrinsic pathways) ($p = 0.010$ for both). Furthermore, strong significant correlations between the caspase-9 levels and BAD and BAX gene expression were observed (Spearman $\rho = -0.76$, $p = 0.011$, and $\rho = -0.72$, $p = 0.011$). **Conclusion:** CRS type 1 induces dual apoptotic pathway activation in monocytes; the two pathways converged on caspase-3. Many factors may induce activation of both intrinsic and extrinsic apoptotic pathways in CRS type 1 patients, such as upregulation of proinflammatory cytokines and hypoxia/

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ischemia. Further investigations are necessary to corroborate the present findings, and to better understand the pathophysiological mechanism and consequent therapeutic and prognostic implications for CRS type 1.

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Introduction

The heart and the kidneys are closely interconnected. Cardiovascular disease may reflect on kidney function and vice versa. Acute or chronic heart failure may lead to a reduction of glomerular filtration rate (GFR), indicating impairment of kidney function, and acute or chronic reduction of GFR may lead to heart decompensation. These findings configure five different types of cardiorenal syndrome (CRS) [1, 2].

CRS type 1 is a multifactorial syndrome characterized by acute deterioration of cardiac function – ischemic (i.e., acute coronary syndrome) or nonischemic (i.e., valve dysfunction) cardiac events – leading to acute kidney injury (AKI). Up to 45% of patients hospitalized for acute decompensated heart failure develop AKI. Low perfusion blood pressure, activation of the neuroendocrine system (sympathetic nervous system and renin-angiotensin-ADH system), and release of vasoactive substances may be involved in AKI development [3]. AKI itself is associated with increased mortality, longer length of hospital stay, and higher risk of chronic kidney disease (CKD) and end-stage renal disease [4].

The pathophysiology of CRS type 1 is complex and involves several factors that are interconnected. Impairment of immune and cellular response, inflammation, endothelial dysfunction, oxidative stress, necrosis, and apoptosis may be involved in the pathogenesis of CRS type 1 [5–10]. Recently, considerable attention has been paid to the role of new alternative mechanisms, such as epigenetics, prenatal programming, small noncoding RNAs, and extracellular vesicles, which may be part of the pathogenesis of cardiorenal cross-talk [11, 12]. Recent data suggest that both tubular epithelial cells and monocytes, when exposed to plasma of patients with CRS type 1, increase cell apoptosis (DNA fragmentation and augmented caspase activity) and cytokine production (IL-6 and IL-18) [7, 12]. Furthermore, higher levels of reactive oxygen species and reactive nitrogen species production were detected in the plasma of CRS type 1 patients when compared to acute heart failure (AHF) patients [6]. In these studies, no severe CKD patients were admitted considering that CKD plasma patients are bearers of factors leading to apoptosis, cellular activation, and inflammation [13, 14]. Overall, these data suggest intensive cross-talk between the heart and the kidneys at cellular and molecular levels. The aim of this study was to confirm the *in vitro* effect of plasma from CRS type 1 patients on a monocyte cell line; in particular, we investigated the caspase pathway mainly involved in apoptotic cascade activation.

Materials and Methods

Study Population

Patients admitted to the Internal Medicine Department of San Bortolo Hospital in Vicenza, Italy, between September 2011 and December 2011 were screened. A total of 65 patients with AHF were further examined for inclusion into the study. AKI was defined by the Acute Kidney Injury Network (AKIN) criteria [15] and CRS type 1 was defined according to the current classification system [1]. Patients with AKI prior to the episode of AHF, patients with other potential causes of AKI or with CKD (estimated eGFR <45 mL/min/1.73 m²), and patients with previous kidney transplantation were excluded. Hypotensive patients who required inotropic support prior to the diagnosis of AKI were not included in the study. We considered as the baseline value the creatinine level of the 3 months before the admission of all patients enrolled into the study.

A total of 40 AHF patients were finally enrolled: 11 of them developed AKI due to AHF during the course of hospitalization and were classified as CRS type 1. AKI was presumably related to cardiac dysfunction after the exclusion of other possible causes of renal damage based on the review of the clinical course of patients. Clinical data, blood pressure, serum creatinine (sCr), urea, hemoglobin, and albumin were evaluated. sCr was measured by the Jaffè method and eGFR was calculated using the 4-variable standardized MDRD study equations.

All procedures were in accordance with the Helsinki Declaration. The protocol and consent form were approved by the Ethics Committee of San Bortolo Hospital. All patients were informed about the experimental protocol and the objectives of the study before providing informed consent and blood samples.

Sample Collection

Blood samples were collected from all 40 patients within 8 h from admission into the Internal Medicine ward. We also collected blood samples within 24 h of AKI for patients who developed CRS type 1. Blood samples were collected into EDTA-containing tubes and processed within 2 h after venipuncture. Samples were subsequently centrifuged for 7 min at 1,600 *g*. After centrifugation, plasma was immediately stored at –80 °C.

U937 Cell Culture

The human cell line U937 is a monocytic precursor cell line derived from a histiocytic lymphoma [16]. The U937 cells were grown in complete liquid phase medium (RPMI 1640; International PBI, Milan, Italy) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin (Sigma Chemical Co., St. Louis, MO, USA). The U937 cells were maintained in a controlled atmosphere (5% CO₂) incubator at 37 °C.

Induction of Apoptosis

U937 cells were treated with plasma from patients with CRS type 1 and AHF. For CRS type 1 patients, we used plasma within 24 h of the AKI event. For AHF patients, we used plasma collected at hospital admission. The ability of plasma to induce apoptosis was evaluated at 24 h. Untreated cells were maintained in the same manner and used as an internal control. U937 cells were plated at 2×10^6 cells per well in 6-well plates, and incubated with 90% RPMI 1640 medium (with 2 mM L-glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin) and 10% of EDTA plasma from the CRS type 1, AHF, and control groups in standard condition (at 37 °C in 5% CO₂ for 24 h). Prior to use, U937 cells were washed twice in Dulbecco's PBS (without calcium and magnesium), pH 7.4. Each incubation was performed in triplicate.

Evaluation of Apoptosis

U937 cell apoptosis was evaluated at 24 h following treatment with CRS type 1 and AHF plasma by cytofluorimetric assay. Cells were washed twice with cold Dulbecco's PBS and suspended in 500 µL of PBS at a concentration of 1×10^6 cells/mL; 100 µL of this solution was incubated with 5 µL of annexin-V FITC conjugated and 2.5 µL propidium iodide (PI) (Beckman Coulter, Brea, CA, USA). The cells were gently vortexed, and incubated for 15 min at room temperature (25 °C) in the dark. Then 400 µL of 1× binding buffer was added to each tube. Analysis was performed by Navios Flow Cytometer (Beckman Coulter) to identify the subpopulations of the apoptotic cells within 1 h. Apoptotic cells were gated and enumerated by identifying those cells that exhibited FITC and PI staining. Annexin-V FITC labeled was used to quantitatively determine the percentage of cells that were undergoing apoptosis. PI was used to distinguish necrotic from nonnecrotic cells. The biparametric analysis shows three distinct populations: viable cells which have low FITC and low PI signals, apoptotic cells which have high FITC and low PI signals, and necrotic cells which have high FITC and high PI signals. A minimum of 20,000 events were collected for each sample.

Determination of Caspases

U937 cells were assayed for activation of caspase-3, caspase-8, and caspase-9 by Enzyme-Linked Immuno-Sorbent Assay (ELISA) Kit (eBioscience, San Diego, CA, USA). U937 cells incubated with plasma for 24 h were processed according to the manufacturer's instructions. Caspase-3, caspase-8, and caspase-9 levels were measured in cell lysates at 450 nm by VICTORX4 Multilabel Plate Reader (PerkinElmer Life Sciences, Waltham, MA, USA). Concentrations of caspases (ng/mL) were calculated from the standard curve according to the manufacturer's protocol.

RNA Isolation, cDNA Synthesis, and RT-qPCR

Total RNA was extracted from 10^6 cells using an RNA isolation kit (ArrowDNA Kit; NorDiag, Holliston, MA, USA) by automatic extractor NorDiag Arrow (NorDiag) according to the manufacturer's protocol. For gene expression analysis, 1 µg of the total RNA was reverse transcribed using random primers (Sigma Aldrich SRL, Milan, Italy) and M-MULV reverse transcriptase (Promega Corporation, Madison, WI, USA). For real-time PCR, GoTaq® qPCR Master Mix (Promega Corporation) was added to appropriate cDNA samples and specific primers for FASL, BAX, and BAD. The PCR was carried out in a total volume of 50 µL including 0.20 µM of forward and reverse primers. Fluorescent data were acquired during each extension phase. Quantitative analysis of gene expression was performed with RotorGene 6000 (Qiagen, Milan, Italy) at a cycle threshold of 0.34 for all samples. Relative expression was determined from cycle thresholds by using individual standard amplification curves of each transcript relative to the corresponding mean expression of two reference transcripts (GAPDH and ACTB).

Statistical Analysis

Statistical analysis was performed using the SPSS Software package. Categorical variables were expressed as percentages; continuous variables were expressed as mean ± standard deviation (parametric variables) or median and interquartile range (IQR) (nonparametric variables). The Mann-Whitney U test or *t* test were used for comparison of two groups, as appropriate. Correlation coefficients were calculated with the Spearman rank correlation coefficient test. A *p* value of <0.05 was considered statistically significant.

Results

Baseline Characteristics of Subjects

AHF was caused by non-ST segment elevation myocardial infarction in 2.5% of patients, excessive salt and fluid intake in 30% of patients, hypertensive crisis in 15% of patients, and other causes in 42.5% of patients. In 10% of patients, no cause of AHF was recognized.

The mean age of 11 patients with CRS type 1 was 76.4 ± 10 years and 45% were males. The median baseline sCr of CRS type 1 patients was 1.06 mg/dL (IQR 0.91–1.3), the median eGFR was 53 mL/min/1.73m² (IQR 48–60). Overall, 7 (63%) CRS type 1 subjects had diabetes and 10 (90%) had hypertension; 27% of patients died during the study period. The mean age of 29 patients with AHF was 73.5 ± 9.8 years and 62% were males. The median baseline sCr of AHF subjects was 1.02 mg/dL (IQR 0.83–1.2), the median eGFR was 66 mL/min/1.73m² (IQR 51–73). Overall, 13 (45%) AHF subjects had diabetes and 27 (93%) had hypertension; 1/29 (3.5%) died during the study period. Characteristics of CRS type 1 and AHF patients are described in Table 1. No patients needed mechanical ventilation and renal replacement therapy. sCr at baseline and albumin were not significantly different in CRS type 1 and AHF patients (*p* = 0.37 and *p* = 0.62, respectively). Moreover, there was no significant difference in terms of median hemoglobin among groups (*p* = 0.06). However, the *p* value did not reach statistical significance; there was a tendency towards a lower level of hemoglobin in CRS type 1 patients. Urea levels were significantly higher in CRS type 1 patients compared to AHF subjects (*p* = 0.012) (Table 1).

Apoptotic Effect of CRS Type 1 Plasma on U937 Cell Apoptosis

The quantitative analysis of apoptosis by flow cytometer confirmed that U937 cells incubated with plasma from CRS type 1 patients had significantly higher apoptosis rates compared to those incubated with plasma from AHF patients (*p* < 0.001). The level of apoptosis detected after 24 h of incubation was 32.0% (IQR 26.8–40.5) for CRS type 1 patients while it was 10.5% (IQR 7.0–14.0) in the AHF group.

The levels of caspase-3, caspase-8, and caspase-9 were measured in U937 cells treated for 24 h with plasma from the two groups. In concordance with the apoptosis rate, U937 cells incubated with plasma from CRS type 1 patients demonstrated a significantly higher caspase-3

Fig. 1. Caspase levels in monocytes exposed to plasma from cardiorenal syndrome (CRS) type 1 and acute heart failure (AHF) patients. The levels of caspase-9 and caspase-8 were significantly higher in monocytes exposed to CRS type 1 compared to AHF.

Table 1. Clinical and laboratory parameters of CRS type 1 and AHF patients included in the study

	AHF with CRS type 1 (n = 11)	AHF (n = 29)	p value
Age, years	76.4±10.0	73.5±9.8	0.53
Weight, kg	77 (67–85)	75 (64–88)	0.84
Male, %	45	62	0.37
Diabetes, %	63	45	0.31
Hypertension, %	90	93	0.91
Death, %	27	3.5	0.12
Serum creatinine at baseline, mg/dL	1.06 (0.91–1.3)	1.02 (0.83–1.2)	0.37
eGFR, mL/min/1.73m ²	53 (48–60)	66 (51–73)	
Urea, mg/dL	73 (55–129.2)	66 (41–81)	0.012
Hemoglobin, g/dL	11.4 (9.7–13)	13.6 (11.1–14.3)	0.06
Albumin, g/L	4.2 (3.94–4.4)	4.13 (4.0–4.33)	0.62
Peak of creatinine, mg/dL	1.43 (1.37–1.59)	0.92 (1.07–1.27)	<0.001

level compared to AHF patients ($p < 0.01$). The increase of apoptosis indicated by flow cytometer was further confirmed by caspase-3 evaluation. In concordance with the apoptosis rate, monocytes incubated with plasma from CRS type 1 patients demonstrated a significantly higher caspase-3 level. The level of caspase-3 detected after 24 h of incubation was 1.98 ng/mL (IQR 1.83–2.11) for CRS type 1 patients while it was 0.79 ng/mL (IQR 0.68–0.98) in the AHF group. A very robust significant correlation between the percentage of monocyte apoptosis detected by cytofluorometric assay and caspase-3 levels was observed (Spearman $\rho = -0.83$, $p < 0.01$).

In addition, we performed a specific analysis to investigate the apoptotic pathway in U937 cells exposed to CRS type 1 plasma. In particular, the level of caspase-8 detected after incubation was 0.58 ng/mL (IQR 0.52–0.74) for CRS type 1 patients while it was 0.42 ng/mL (IQR 0.33–0.53) in the AHF group ($p = 0.003$) (Fig. 1). The level of caspase-9 was 72.3 ng/mL (IQR 66.9–94.5) for CRS type 1 patients while it was 27.1 ng/mL (IQR 22.8–37.8) in the AHF group ($p < 0.01$) (Fig. 1). Both caspases were significantly higher in CRS type 1 compared to

Fig. 2. Concept figure of apoptosis activation in monocytes exposed to cardiorenal syndrome (CRS) type 1 plasma.

Table 2. Gene expression analysis for BAD, FASL, and BAX in monocytes treated with CRS type 1 and AHF plasma

	AHF with CRS type 1	AHF	p value
BAD	39.3 (26.4–50.5)	6.4 (5.1–8.1)	0.010
FASL	34.9 (4.0–8.5)	66.1 (28.7–78.7)	0.762
BAX	597.5 (414.7–786.5)	136.4 (129.1–191.9)	0.010

AHF. In addition, we observed a strong significant correlation between caspase-3 and caspase-9 levels ($\rho = -0.66$, $p < 0.01$) and a significant correlation between caspase-3 and caspase-8 ($\rho = -0.33$, $p = 0.037$).

To gain further insight into the mechanisms underlying the initiation of both pathways of apoptosis in monocytes exposed to CRS type1 plasma, pathway-focused PCR arrays related to apoptotic mechanisms were performed. In particular, we investigated the expression of FASL, BAX, and BAD. BAD and BAX gene expressions were significantly higher in monocytes treated with CRS type 1 plasma compared to AHF ($p = 0.010$ for both). On the contrary, FASL expression was similar in monocytes treated with CRS type 1 and AHF plasma ($p = 0.76$). In particular, there was a significant increase in the expression of BAX and BAD genes by 6- and 4- respectively in U937 cells treated with CRS type 1 plasma compared to those treated with AHF plasma (Table 2). Furthermore, strong significant correlations between caspase-9 levels and BAD and BAX gene expression were observed ($\rho = -0.76$, $p = 0.011$, and $\rho = -0.72$, $p = 0.011$).

Discussion

The pathophysiology of CRS type 1 is multifaceted and poorly understood and it involves several factors. In particular, recent studies have demonstrated a pathogenic role of apoptosis in the development of AKI and AHF, and in CRS type 1 initiation [8, 17, 18]. Activation of different apoptotic pathways is supposed to have an essential role in the mechanism of CRS type 1 and it could be a potential therapeutic target in this syndrome. Two main intracellular pathways have been recognized as apoptotic mechanisms: ligation of plasma membrane death receptors (extrinsic pathway) and perturbation of intracellular homeostasis (intrinsic pathway).

In this study, we confirmed a marked proapoptotic activity in monocytes incubated with CRS type 1 plasma. Furthermore, we reported higher levels of caspase-3, caspase-8, and caspase-9, and a significant direct correlation between effector caspase (caspase-3) and caspase-8 and caspase-9 levels. These data have revealed a strong activation of apoptosis through intrinsic (via caspase-9 activation) and extrinsic (via caspase-8) pathways in monocytes exposed to CRS type 1 plasma. Both caspase-9 and caspase-8 converge on caspase-3 activation leading to DNA fragmentation, cytoskeletal demolition, cross-linking of protein, and formation of apoptosis bodies cleared by phagocyte cells [19]. Based on our results, we present a concept figure demonstrating activation of the intrinsic and extrinsic pathways at the level of monocytes in CRS type 1 (Fig. 2).

In previous works, we showed the strong upregulation of plasma proinflammatory cytokines, such as IL-6 and TNF- α , in CRS type 1 patients [17]. Loss of normal balance of the immune system associated with strong oxidative stress may be involved in the altered immune regulation that causes a defective regulation of monocyte apoptosis as well as tissue and organ damage [6, 9, 17]. All these factors may play a crucial role in kidney dysfunction and in the progress of AHF [17]. In this context, it is known that proinflammatory cytokines lead to cellular apoptosis through activation of the extrinsic pathway, via death domain activation [7, 17].

Relative hypovolemia during acute decompensated heart failure and consequent hypoperfusion of nephron, reduction of kidney oxygen delivery, and impairment of cellular aerobic metabolism may be implicated in the upregulation of apoptosis through activation of the intrinsic and extrinsic pathways, as well as stimulation of the inflammatory system [20–22]. Furthermore, in CRS type 1 patients we observed a tendency of lower levels of hemoglobin compared to AHF subjects. This trend may also explain relative hypoxia in CRS type 1 patients, leading to increased apoptotic processes. It is well known that hypoxia causes a reduction in ATP production, alteration of plasmatic and mitochondrial membrane permeability, intracellular ionic imbalance, and finally, cell death by necrosis and apoptosis [23].

To confirm our results obtained from caspases analysis, we investigated the gene expression of FASL, BAX, and BAD. In the extrinsic pathway, the FAS/FASL system transmits apoptotic signals from the surrounding environment into the cell; the binding of FASL with FAS initiates receptor oligomerization, which recruits FAS-associated death domain and activates caspase-8 and caspase-10 [24–26]. BAX and BAD are proapoptotic members, which are involved in initiating apoptosis in the intrinsic pathway [27]. Our gene expression results described a significant increase in BAX and BAD expression in monocytes exposed to CRS type 1 plasma. These data confirmed and demonstrated a strong and abnormal activation of the intrinsic pathway in apoptotic monocytes. Specifically, BAX and BAD are involved in outer mitochondrial membrane permeabilization allowing soluble proteins, such as cytochrome c, to diffuse into the cytosol and activate the caspase cascade [28]. Our results are similar to a recent report demonstrating dual activation of the apoptotic pathway, by upregulation of caspase-3, caspase-8, and caspase-9 in renal tubular cells incubated with CRS type 1 plasma, compared to AHF plasma [29, 30].

Based on these results, we hypothesized that caspase may be a target for new therapy. In vivo caspase inhibitors protect against ischemic injury in different tissues such as brain, heart, and kidney [31]. These interventions may prevent AKI and progress of AHF, avoiding CRS type 1. These are further explanations of the pathogenic role of apoptosis in renal damage during CRS type 1. Additionally, we speculated that the interaction FAS/FASL could be a target for an innovative specific therapy. In this context, Hamar et al. [32], in an in vivo mouse model of renal ischemia-reperfusion injury, reported the prevention and the reduction of renal damage after administration of small interfering RNA (siRNA) targeting FAS. However, in our study, we found only significant increased levels of Caspase-8 and we did not observe FASL up-regulation in monocytes exposed to CRS 1 type. This result could be related to the small sample size analyzed. Actually, we have not enough evidence to completely understand all mechanisms involved in the pathogenesis of CRS Type 1. We speculated a pivotal role of inflammation and apoptosis dysregulation in CRS Type 1 initiation and development.

In the next future, the multi-step inhibition of apoptosis may be object of scientific research in CRS Type 1. We can theorize that the inhibition apoptosis process at different steps using both pharmacological and genetic approaches, to either prevent or minimize organ and tissue ischemic damage.

In conclusion, in this study, we investigated the role of apoptosis in CRS Type 1 pathogenesis. CRS Type 1 induces dual apoptotic pathway activation in monocytes; the two pathways converged by caspase-3. In particular, we found up-regulation of both intrinsic and extrinsic pathway of apoptosis as well as demonstrated by significant increase of both caspase-8 and -9 levels. Furthermore, we also found over-expression of both Bcl-2 family members BAX and BAD genes, implicated in intrinsic pathway of apoptosis. Many factors may act on the activation of both apoptotic pathways such as pro-inflammatory cytokines and hypoxia/ischemia; these last factors may be related to the hypovolemia causing hypoperfusion of nephron. There has been growing interest in organ-organ interaction as a way of understanding the underlying pathophysiology of concomitant heart and renal dysfunction. The complexity of CRS Type 1 presents a key challenge for singular diagnostic or treatment approaches. Further investigations are necessary to corroborate the present findings and to better understand pathophysiological mechanism and consequent therapeutic and prognostic implications for CRS Type 1.

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Statement of Ethics

This study was approved by the Ethics Committee of San Bortolo Hospital in Vicenza and the procedures were in accordance with the Helsinki Declaration.

Disclosure Statement

The authors declare no conflict of interest.

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