PRO-INFLAMMATORY CYTOKINES ELICIT INFLAMMATORY RESPONSE IN BLOOD LEUKOCYTES OF POST DIALYTIC CHRONIC RENAL PATIENTS THROUGH HEME OXYGENASE-1 ACTIVATION

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ABSTRACT

Objective: Inflammation is common in patients with chronic renal failure and has been associated with the increased production of pro-inflammatory cytokines. The aim of the investigation is to assess the levels of the pro-inflammatory cytokines, TGF-β, IL-1β and TNF-α and correlate it with HO-1 expression in the leukocytes of renal failure patients.

Methods: Cytokine profile was analyzed in 53 South Indian patients suffering from ESRD under post dialysis condition (2 hrs after dialysis).

Results: Augmented production of TGF-β, IL-1β and TNF-α was observed in the peripheral blood leukocytes of the patient population. An increase in the expression of HO-1 in the leukocytes was demonstrated from our earlier studies.

Conclusion: Thus, our study hypothesized that the inflammatory reaction in the leukocytes of the post dialytic renal failure cases might be due to the elevation in HO-1 levels brought about by altered pro-inflammatory cytokine levels.

Keywords: Pro-inflammatory cytokines, HO-1, Dialysis, Leukocytes, Renal failure, Blood.

INTRODUCTION

Chronic kidney disease (CKD) is a heterogeneous condition, and the clinical manifestations, progression and management depend on its cause, pathology and other comorbid conditions. The most serious adverse outcomes of CKD include metabolic complications like decreased glomerular filtration rate, progressing to endstage renal failure (hypertension, anemia, malnutrition, bone and mineral disorders, etc.) and increased risk for cardiovascular diseases [1]. Chronic inflammation appears to underlie most of the chronic diseases of today, including Cardiovascular disease, type 2 diabetes, chronic kidney disease, Alzheimer’s disease and cancer[2]. Studies consistently showed that CKD is an inflammatory process and that the biomarkers of inflammation increase since early stages of CKD [3]. Compelling evidence suggests that inflammation is a major factor in renal ischemia/injury [4]. Elevations in both pro-inflammatory cytokines [e.g., tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6)] and anti-inflammatory cytokines [e.g., interleukin-10 (IL-10)] have been demonstrated in humans with renal failure [5]. A growing body of evidence suggests that over expression of hemeoxygenase-1 (HO-1) may protect organs/tissues from immune-mediated injury either through prevention of oxidative damage or via a local immunomodulatory influence on infiltrating inflammatory cells [6]. Studies indicate that a deficiency of HO-1 may predispose to generally exaggerated inflammatory responses, suggesting that its activity is necessary for timely resolution of early inflammation.

Wei-Xia et al, 2007 [7], postulated that induction of HO-1 could augment IL-10 and transforming growth factor (TGF-β) production in Treg cells thereby leading to attenuation of airway inflammation. Also, over expression of HO-1 was found to protect against, TNF-α mediated airway inflammation by down-regulation of TNFR1-dependent oxidative stress [8]. We previously reported that HO-1 expression was found to be highly elevated in the peripheral blood leukocytes of post dialysis renal failure patients[9]. However, this augmentation in HO-1 expression did not ease the burden of the disease condition. Despite these observations, very little is known about the specific mechanisms involved in HO-1-mediated regulation of the immune response. Hence, the aim of the present investigation is to determine the levels of the pro-inflammatory cytokines, such as, TNF-α, IL-1β and TGF-β and their correlation with the HO-1 activity in the lymphocytes of CRF patients. This study will help to determine the role of the cytokines in renal failure patients. Thereby leading to the better understanding of the mechanisms involved in HO-1 up regulation in renal failure patients.

MATERIALS AND METHODS

Chemicals

Mouse monoclonal IgG transforming growth factor-beta-1 [Cat no. sc-65379β], Rabbit polyclonal Interleukin-1 beta [Cat No. sc-78894β] and Goat polyclonal IgG TNF-α [Cat No. sc-13513β] were purchased from Santa Cruz Biotech USA. Secondary antibodies (Goat anti-Rabbit and Rabbit anti-Goat and Mouse anti-human) were procured from Bangalore Genei. Diaminobenzidine (DAB) was obtained from Himedia, India. 4, 6-diamidino-2-phenylindolehydrochloride (DAPI) was purchased from Qualigen, Germany. Forward and reverse primers for amplification of TGF β and GAPDH were obtained from Bangalore Genie, India. All other chemicals used were of analytical grade.

Study population

The study population comprised of two groups of individuals. Blood samples was collected from 53 South Indian patients with chronic renal failure (creatinine levels >5mg/dl) and classified under End Staged Renal Disease from Stanley Hospital, Chennai, India. Exclusion criteria for participation in this study were acute infections, malignant diseases, cardiovascular events (myocardial infarction, stroke) and diabetic nephropathy.

Group I Normal healthy volunteers served as controls (n= 50); Group II individuals suffering from CRF served as patients (n=53). The membrane commonly used for dialysis is polyarylethersulfone. The composition of the dialysate is sodium chloride, sodium bicarbonate or sodium acetate, calcium chloride, potassium chloride, magnesium chloride and rarely glucose. The study was approved by Stanley Medical College and Hospital and The Institutional Ethical
Committee. Consent was obtained from all participants before enrollment.

**Lymphocyte isolation**

Peripheral blood leukocytes (PBL) were isolated from whole blood by the method of Hashi et al. 1998[10].

**Immunocytochemical identification of IL-1β and TNF-α**

Immunocytochemical analysis of the cytokines was performed by the method of Berod et al. 1981[11]. The cells were fixed either in ice-cold methanol, acetone (1-10 min) or in 3-4% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature. Washed twice with ice cold PBS. Cells incubated for 10 min with PBS containing 0.25% Triton X-100 and washed again in PBS three times (5 min). Further the cells were incubated with 1% BSA in PBS-T for 30 min to block unspecific binding of the antibodies. The cells were treated with diluted antibody in 1% BSA in PBS-T in a humidified chamber for 1 hr at room temperature and washed again in PBS three times (5 min). The smear was incubated with the secondary antibody in 1% BSA for 1 hr at room temperature in dark. Washed again in PBS three times (5 min) and visualized in immunofluorescence microscope.

**Amplification of TGF-β gene by Reverse transcription-Polymerase chain reaction (RT-PCR) Isolation of RNA**

All the reagents were prepared under ice-cold conditions and all the glasswares used were made sterile with diethyl pyrocarbonate (DEPC) (0.1 %) treated water. Whole blood was transferred to a fresh tube, an equal volume of isopropanol was added and the samples were stored for 45 min at -20°C. The samples were centrifuged in 12,000 rpm for 15 min at 4°C and the RNA precipitated was sedimented as an invisible pellet at the bottom of the tube. The supernatant was removed; the RNA was washed twice with 75% ethanol by vortexing and subsequently centrifuged for 8 min at 12,000 rpm (4°C). DEPC treated and boiled RNAase free solution was used for RNA solubilization. The integrity of the solution was checked spectrophotometrically with a 260/280 ratio showing absorbance higher than 2.

**Primers for cDNA synthesis and gene amplification**

The single strand cDNA was prepared in all the samples and was used as a template for preparing the following gene fragment amplification. The reaction mixture contained 2 µl of 10x reaction buffer, 50 mM KCl and 10 mM Tris HCl (pH 8.3). Primers were used at a final concentration of 10 picomoles. The four-deoxynucleotide triphosphates (dATP, dGTP, dCTP and dTTP) were used at a concentration of 2 mM each.

**cDNA (complementary DNA) synthesis**

500 ng of RNA was used for the synthesis of the single strand cDNA for all the samples. 50 mM of oligo (dT) (12-18) was added to quench the RNA and this was performed at 65°C for 5 min. After quenching, 2 µl of assay buffer (10x), 2 µl of dNTP’s 10 mM and 0.25 µl of M-MulV-RT (25U) were added to each tube and the required amount of DEPC treated water was added to make the reaction volume to 20 µl. The reaction was performed at 42°C for 10 min and at 37°C for 1 h in the PCR instrument (TECHNE, T echgene, UK). The products were analysed in 1.5% agarose gel to observe the constructed single strand cDNA and this was used as a template for the second round of PCR. The following reagents were prepared in the given ratio for 20 µl reaction.

**Table 1: Primer sequence constructed from the gene bank nucleotide sequence of specific gene of interest as(sense) and (antisense)**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>TGF-β</td>
<td>5’-GCCCTGGGAGCCAACATTGC-3’</td>
<td>5’-CTCAGTGGCATGGCCCTTC-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-CCACACCCCTGTGTCTCTAG-3’</td>
<td>5’-GGCACTGGCAGAGGCAA-3’</td>
</tr>
</tbody>
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PCR conditions were as follows: 4 minutes at 95°C, 1 minute at 58°C, and 35 seconds at 72°C (32 cycles).

**Table 2: Preparation of reagents for PCR**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Reagents</th>
<th>Quantity</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Template cDNA</td>
<td>100ng</td>
<td>1 µl</td>
</tr>
<tr>
<td>2</td>
<td>Primers Forward</td>
<td>10pM</td>
<td>1 µl</td>
</tr>
<tr>
<td>3</td>
<td>Reverse</td>
<td>10pM</td>
<td>1 µl</td>
</tr>
<tr>
<td>4</td>
<td>M asternk</td>
<td>2X</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>5</td>
<td>Mill Q water</td>
<td>------</td>
<td>4.5 µl</td>
</tr>
<tr>
<td></td>
<td>Total Reaction</td>
<td></td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Each of the above reagents was added to each of the tube and the required amount of Milli Q water was added to make the reaction volume to 20 µl.

After performing the experiment, the PCR products were visualized in a 1.5% agarose gel under UV light using the gel documentation system. DNA molecular weight ladder of 1000 bp was also run along with the product to check the product size.

The intensity of expression of the product was observed and compared.

**Dot blot procedure**

The nitrocellulose membrane is made ready by drawing the grids. About 2 µl of sample was spotted onto the nitrocellulose membrane at the center of the grid. The membrane is allowed to dry. Non-specific sites are blocked by soaking in 5% BSA in TBS-T (30 mins-1 hr, RT). The membrane was incubated with primary antibody.
RESULTS
Role of TNF-α in activation of immune reactions
Monocyte activation and the synthesis of proinflammatory cytokines lead to self-propelling reactions of synthesis and release of anti-inflammatory receptors and cytokines. In our study, we preferred to determine the degree of inflammation in the cells by investigating the expression of 3 key pro-inflammatory cytokines such as TNF-α, IL-1β and TGF-β. TNF-alpha, a key cytokine in innate immune responses is increased during lesions and has many effects, ranging from inflammation to apoptosis, and is a key apoptotic marker. In addition, it can also trigger the production of various regulatory factors, including HO-1. Figure 1 illustrates the immunocytochemical analysis of TNF-α in the control and renal failure patients respectively. An elevated expression of TNF-α was observed in the patient sample as compared to the control, indicating inflammation, which might be due to apoptotic condition in the patients.

IL-1β and TGF-β in inflammation
The other major cytokine which has a significant role in inflammation is IL-1β and it is not only a key proinflammatory cytokine, but also found to activate HO-1. In this context, immunocytochemical analysis of IL-1β was performed to study its expression in the leukocytes and thereby correlate with HO-1 production. Figure 2 represents the Immunocytochemical profile of IL-1 β in the normal and CRF patients. The expression of IL-1β was found to be significantly increased in the patient group as compared to the control group. This is further confirmed by dot blot analysis of IL-1β in control and patient's group.

A number of investigations have demonstrated that TGF-β plays a pivotal role in the progression of renal diseases by driving inflammatory reactions, and it was found to be an excellent stimulator of HO-1 in various cell lines. To study its role, we determined its expression by RT-PCR analysis and dot blot analysis. Figure 3 shows the RT-PCR pattern of TGF-β in the control and CRF patients. The expression of TGF-β was found to be significantly upregulated in the patient sample, indicating its participation in inflammation. Further analysis using dot blot also corroborates the results obtained from RT-PCR analysis.

Our previous reports have shown the enhanced expression of HO-1 in the leukocytes of the renal failure patients undergoing treatment (dialysis), with the maximum expression attained during post dialysis condition, which could possibly be due to high grade of...
inflammation. The results obtained in our investigation, could attribute to the possible involvement of IL-1β, TGF-β and TNF-α in elevating leukocyte HO-1 expression during post dialysis conditions in the patient population, thereby leading to severe inflammatory reactions.

TNF-α is a pleiotropic cytokine which plays important role in renal diseases such as lupus nephritis, anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis and renal allograft rejection. However, TNF-α also plays critical immunoregulatory roles that is required to maintain immune homeostasis [14]. Nakamishi et al, 2004 [15], showed that hemodialysis patients presented a significant increase in serum levels of IL-8, IL-6 and TNF-α compared to normal individuals. Likewise, Kir et al, 2012 [16], measured serum TNF-α levels in chronic renal failure cases and found it to be highly elevated. Wang et al., 2008 [17], have reported that ESRD patients, irrespective of whether treated or not treated by dialysis, had high levels of plasma IL-1β, IL-6, TNF-α, and CRP in dialysis patients. These elevations should confer increased cardiovascular risk of ESRD patients on dialysis.

HO-1 induction is considered to be an adaptive cellular response to survive exposure to environmental stresses. Although the function of this enzyme is not completely understood, increasing evidence suggests that HO-1 plays a vital role in many aspects, such as suppression of oxidative stress, inflammation, cell proliferation, microcirculation improvement and regulation of cytokine expression in various pathological conditions [18]. Although pre-induction of HO-1 inhibits inflammation, pro-inflammatory mediators like TNF-α, IL-1, LPS and oxidized lipids are found to be potent inducers of HO-1 expression in endothelial cells and macrophages phages [19].

Not much research has been performed on HO-1 activity/levels in leukocytes isolated from renal failure cases. Our previous studies have demonstrated increased expression of HO-1 in renal failure cases, with the maximum observed in post dialysis condition [20].

In the present investigation elevated expression of TNF-α by immunocytochemical analysis was confirmed. Thus proving enhanced rate of inflammation in the patients, which suggests its possible role in HO-1 activation and is in agreement with earlier reports. IL-1β is a highly inflammatory cytokine and has been shown to be associated with protein catabolism in several chronic disease states, including advanced uremia [21]. The release of IL-1β, IL-6 and TNF-α stimulates lymphocytes for synthesis of IL-2 [22]. Faubel et al, 2007[23], demonstrated that cisplatin-induced acute renal failure is associated with an increase in the cytokines IL-1β, IL-18, and IL-6 and neutrophil infiltration in the kidney.

Although pre-induction of HO-1 inhibits inflammation, pro-inflammatory mediators such as LPS induce the expression of HO-1 [24]. Terry et al, 1999, also demonstrated that IL-1β-induced NO production in human endothelial cells [24]. Faubel et al, 2000 [25], demonstrated that the anti-inflammatory effects of LPS and IL-1β were found to stimulate HO-1 expression in human endothelial cells [24].

Fig. 3: RT-PCR electrophoretic pattern densitometric analysis and dot blot analysis of TGF-β levels in the control and patient group respectively

Lane 1 to 4 CRF patient sample showing enhanced TGF-β mRNA expression

Lane 5 Control sample showing decreased expression of TGF-β mRNA

Lane 6 1000 bp Marker

Results are expressed as mean±SE (n=53) *** p<0.001 between Group 1, control; group 2, CRF Patients. $-$ represents the significance in comparing the control and patient group.

DISCUSSIONS

Prevalence of inflammation is high in CKD patients, as reflected by elevated levels of acute phase reactants such as serum C-reactive protein (CRP), and other proinflammatory cytokines [12]. It has been recognized that about 30-50% of non-dialedyzed, hemodialysis (HD), and peritoneal dialysis (PD) patients have serologic evidence of active inflammatory response. Peripheral monocytes infiltrating the kidney have traditionally been considered the primary source of renal TNF; however, evidences suggest that glomerular mesangial cells are an important additional source [13].

Extensive studies have demonstrated that TGF-β plays an important role in the progression of renal diseases. Ning et al, 2002 [28], first determined that TGF-β is capable of strongly inducing the expression of HO-1 in human pulmonary epithelial cells (A549). The levels of both HO-1 mRNA and HO-1 protein increased upon stimulation with exogenous TGF in A549 cells. Kutty et al, 1994 [29], previously demonstrated that TGF-β induced HO-1 mRNA and protein levels in human retinal pigment epithelial cells and bovine choroid fibroblasts. In the present study, an increase in the expression/levels of TGF-β was observed in renal failure cases by...
both RT-PCR and dot blot analysis. This indicates that TGF β has a potential role to play in inflammation in the CRF patients. The results can be corroborated with previous reports of TGF β induction of HO-1 in these patients.

CONCLUSION

In the present investigation, CRF patients exhibited elevated TNF α expression, which could be probably due to the cell’s response to stress induced by the disease. Thus, TNF α might serve as an important factor in activating HO-1 levels in the patient group. A rise in the levels of the crucial pro-inflammatory cytokines, TGF β and IL-1 β, in the CRF patients, implies their active participation in the process of inflammation. Since such pro-inflammatory cytokines have a key task to play in HO-1 stimulation, as observed from the previous reports, it can be considered that TNF α, TGF β and IL-1 β have an unambiguous role in HO-1 activation. Activation of HO-1 expression plays a central role during the progression of chronic renal failure. Hence, the designing of a therapeutic strategy targeted towards the regulation of HO-1 expression might create a new genre of drugs for treatment of renal failure.

CONFLICT OF INTERESTS

Declared None

REFERENCES