

Hydrocortisone Reduces Toll-Like Receptor 4 Expression on Peripheral CD14⁺ Monocytes in Patients Undergoing Percutanous Coronary Intervention

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ABSTRACT

Background: Evidence from several lines of investigations suggests that Toll-like receptor 4 (TLR4) is involved in atherosclerosis as a bridge between innate and acquired immunity. Percutaneous coronary intervention (PCI) can trigger inflammation through activation of human TLR4 (hTLR4) on monocytes. Hydrocortisone as an anti-inflammatory and immuno-suppressant agent has multiple mechanisms of action. In this study, we aimed at assessing the effects of hydrocortisone on monocyte expression and activity of hTLR4 in patients underwent PCI. **Methods:** Blood samples were taken from a total of 71 patients with chronic stable angina who were scheduled for a PCI, before the intervention. Thirty patients received 100 mg hydrocortisone prior to the procedure. Control group was composed of 41 patients underwent PCI without receiving hydrocortisone. Blood collection was repeated 2 and 4 h after PCI. The expression of hTLR4 on the surface of CD14⁺ monocytes and the serum levels of TNF- α and IL-1 β were measured using flowcytometry and Sandwich ELISA. **Results:** Compared with controls, hydrocortisone significantly reduced monocyte expression of hTLR4 in test group ($P < 0.01$). In addition, it had a significant effect on reduction of serum concentrations of TNF- α and IL-1 β in test group in a time-dependent manner ($P < 0.01$). **Conclusion:** In this study, hydrocortisone was able to reduce the hTLR4/CD14 positive monocytes and its related pro-inflammatory cytokines, thus it can decrease inflammatory responses following PCI. *Iran. Biomed. J. 18 (2): 76-81, 2014*

Keywords: Toll-like receptor 4, Cytokines, Hydrocortisone

INTRODUCTION

Inflammation and immune cell activation are well-established processes in ischemic heart diseases. A considerable amount of evidence links the mechanisms underlying the initiation of coronary plaque development and disruption with inflammation [1-3]. A large body of interest has continuously centered on understanding the role of cellular and molecular components of inflammation involved in coronary artery diseases (CAD). Toll-like receptors (TLR), a class of pattern recognition receptors, have emerged as bridges between innate and adaptive immunity [4]. Most mammalian species have between 10 and 15 types of TLR. Ten functional TLR have been identified in human [5]. Among them, human

TLR4 (hTLR4) has gained prominence in researches related to atherosclerosis and cardiovascular disorders [6]. hTLR4 can be activated by a number of exogenous and endogenous ligands such as bacterial lipopolysaccharides, fungi [7], heat shock protein 60 and 70 [8], minimally modified LDL and oxidized LDL [9]. hTLR4 has considerable expression on leukocytes including CD14⁺ monocytes and dendritic cells [10]. Its activation is followed by interaction with an adaptor protein, myeloid differentiation factor (MyD88), and translocation of nuclear factor kappa B (NF- κ B) to nucleus which eventually leads to production of pro-inflammatory cytokines, such as TNF- α , IL-1- β , and IL-6 [11, 12]. Pro-inflammatory cytokines in the myocardium and peripheral tissues have been identified as important

markers of myocardial dysfunction [13, 14]. Glucocorticoids are widely recognized as anti-inflammatory agents that are of immense value in suppressing immunity. The immuno-suppressive and anti-inflammatory actions of glucocorticoids are inextricably tied together; perhaps they both involve inhibition of leukocyte functions [15]. Revascularization therapies such as percutaneous coronary intervention (PCI) are applied for patients with stable angina who are refractory to medical therapy [16]. We previously reported that PCI could trigger inflammatory responses through up-regulation of monocyte expression of hTLR4 and elevation in serum levels of TNF- α and IL-1 β [17]. Hydrocortisone is used prior to PCI procedure to prevent allergic reactions due to contrast materials. However, its administration is not conventionally performed in all of the PCI procedures. Previous studies have not adequately assessed the effects of different drugs on expression and activity of inflammatory components in PCI setting [6, 16]. We hypothesized that a part of anti-inflammatory effects of hydrocortisone is mediated through suppression of hTLR4 expression and activity. The present study was designed to investigate the effects of hydrocortisone on the expression of hTLR4 on circulating CD14⁺ monocytes in patients underwent PCI and also to explore its effect on hTLR4 downstream signaling products.

MATERIALS AND METHODS

Study patients. We studied 30 patients with stable angina, who were scheduled for an elective PCI, in Shahid Madani Heart Hospital, Tabriz, Iran. The exclusion criteria were as follows: previous myocardial infarction within 6 months, autoimmune diseases, inflammatory conditions, advanced hepatic or renal disease, and malignant neoplastic diseases. None of the patients had valvular heart disease. Cardiovascular risk factors, medications, sex, age, and previous medical history were obtained by a questionnaire and by medical records of the patients. All PCI procedures were carried out according to protocols of the hospital. White blood cell count, cholesterol, glucose, prothrombin time, partial thromboplastin time, blood urea nitrogen, creatinine, sodium, and potassium were measured according to routine protocols. Control group was composed of 41 patients with stable angina who underwent PCI without receiving hydrocortisone. The study was approved by the local ethical committee, and an informed consent letter was obtained from all participants.

Blood collection and processing. A total of 6 ml blood was taken via antecubital venipuncture at time of

admission (0h). Of 6 ml blood, 2 ml was kept in sterile EDTA bottles for flowcytometry analysis and 4 ml was centrifuged (3000 \times g for 5 min) to obtain serum. Then, the serums were kept in -80°C. Blood collection was repeated 2 and 4 h after termination of PCI procedure.

Stenting procedure. Test and control groups received intravenous heparin (10,000 U) before the stenting procedure. Hydrocortisone succinate (100 mg, Rotexmedica, Trittau, Germany) was intravenously administered into test group 30 min before the procedure. All individuals took aspirin (80 mg) and clopidogrel (75 mg) per day.

Flowcytometry analysis. Briefly, cells were stained at 4°C for 30 min with monoclonal antibodies for human CD14 (Abcam, Cambridge, UK) conjugated with FITC and hTLR4 (Abcam) conjugated with phycoerythrin. FITC and phycoerythrin-conjugated non-specific mouse IgG2a antibodies were used for isotype controls (Abcam). Cells were washed, and cell-associated fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA). Data were analyzed by CellQuest software (BD Biosciences).

Measurement of TNF- α and IL-1 β serum levels by ELISA. For measurement of TNF- α and IL-1 β serum levels, a Sandwich ELISA was performed (Ray Biotech, Norcross, Georgia, US). In short, 100 μ l serum was added to microtiter plates. The incubation time was at room temperature for 2.5 h. After that, 100 μ l prepared biotinylated antibody and streptavidin solution were added to each well and incubated at room temperature. The intensity of the color was measured at 450 nm by Stat Fax 2600 (Awareness Technology, Palm City, Florida, USA) plate reader. Stat Fax 2100 (Awareness Technology) was used for washing steps.

Statistical analysis. Data are reported as mean \pm SD or numbers (percentage). Means were compared by Wilcoxon signed-rank test. Differences at the level of $P < 0.05$ were considered statistically significant. All analyses were performed using SPSS 16.0.

RESULTS

Characteristics of patients. Table 1 shows clinical characteristics of studied patients. No significant difference was observed in gender, age, and use of medications, including aspirin, beta-blockers, statins, angiotensin-converting enzyme inhibitors, nitroglycerin or blockers of angiotensin receptor 2, white blood cell count or the risk factors among individuals.

Table 1. Clinical characteristics of the subjects in the study

Characteristics	Control (n = 41)	Test (n = 30)
Age, years	58 ± 9.7	54 ± 8
Male	28 (68.3)	21 (70)
Hypertension	20 (48.8)	21 (70)
Diabetes	22 (53)	24 (80)
Hyperlipidemia	11 (26.8)	11 (36.6)
Familial history	9 (22)	10 (33.3)
Smoking	13 (31.7)	11 (36.6)
Medications		
Nitrates	21 (51.2)	21 (70)
β blockers	27 (65.9)	27 (90)
ACE inhibitors	19 (46.3)	14 (46.6)
α2 blockers	13 (24.07)	4 (13.3)
Calcium channel blockers	7 (17.1)	5 (16.6)
Statins	35 (85.4)	28 (93.3)
ASA	38 (92.7)	26 (86.6)
Laboratory parameters		
C- reactive protein (mg/dl)	0.4 ± 1.7	0.4 ± 1.3
Cholesterol (mg/dl)	190 ± 40	188 ± 37
LDL (mg/dl)	141 ± 31	141 ± 21
HDL (mg/dl)	42 ± 6	41 ± 9
Glucose (mg/dl)	111 ± 23	97 ± 21
White blood cell count (U/L)	7.8 ± 0.2	7.1 ± 0.4

Data are shown in number (%) or mean ± SD; ACE, angiotensin converting enzyme; ASA, acetyl salicylic acid; Angiotensin receptor 2 (A2 blockers)

Expression of hTLR4 on CD14 positive monocytes.

As assessed by flowcytometry, monocytes showed surface staining for hTLR4 and the monocyte marker CD14 (Fig. 1A and 1B). The percentage of CD14 positive monocytes containing hTLR4 was 23.3 ± 2.7 in control group and 29.3 ± 2.1 in test group at baseline. In controls, hTLR4⁺/CD14⁺ monocytes were $37.2 \pm 3.7\%$ and $32.9 \pm 3.3\%$, respectively 2 and 4 h after PCI. A significant reduction in hTLR4⁺/CD14⁺ monocytes was seen in test group 2 and 4 h after PCI ($15.1 \pm 3.7\%$ and $14.3 \pm 3.3\%$, respectively, $P < 0.01$, Fig. 1C) as compared to control group.

Serum levels of TNF-α. As presented in Table 2, serum concentration of TNF-α significantly was reduced significantly to 13.4 ± 2.7 (pg/ml) and 11.7 ± 1.6 (pg/ml) 2 and 4 h after PCI, respectively as compared to controls.

Serum levels of IL-1β. Two and 4 h after PCI, a significant reduction of IL-1β was seen in serum (9.1 ± 2.3 and 8.5 ± 1.4 (pg/ml), respectively) (Table 3).

DISCUSSION

In the present study, we showed, for the first time, that hydrocortisone was able to reduce the expression of hTLR4 on CD14⁺ monocyte and decrease serum levels of TNF-α and IL-1β in patients who underwent PCI compared to those who did not received this drug. Our

results suggest that hydrocortisone suppresses inflammatory responses following PCI by inhibiting the monocyte expression of hTLR4⁺/CD14⁺. It also causes a considerable reduction in serum levels of pro-inflammatory cytokines. Several mechanisms are involved in the suppression of inflammation by glucocorticoids. Direct physical association between activated glucocorticoid receptors and NF-κB inhibits transcription activity. These protein-protein cross talks have been observed between the glucocorticoid receptors and NF-κB, which regulates the expression of many components of the immune system. Glucocorticoids can up-regulate the cytoplasmic inhibitor of NF-κB, I-κB and thus inhibit translocation. In addition, glucocorticoids have inhibitory effects on genes for inducible nitric oxide synthase, cyclooxygenase-2, and inflammatory cytokines [15, 18, 19]. These results highlight the distinguished role of glucocorticoids as anti-inflammatory and immunosuppressive agents. Immune system activation and inflammation are widely involved in the development and progression of CAD [20]. Therefore, markers of

Table 2. Serum levels of TNF-α (pg/ml) at different time intervals

Group	Time intervals (h)		
	0	2	4
Control (n = 41)	23.5 ± 3.7	35.4 ± 5.1	34.2 ± 3.3
Test (n = 30)	21.5 ± 2.2	13.4 ± 2.7*	11.7 ± 1.6**

* $P < 0.01$ vs. control group at 2 h; ** $P < 0.01$ vs. control group at 4 h

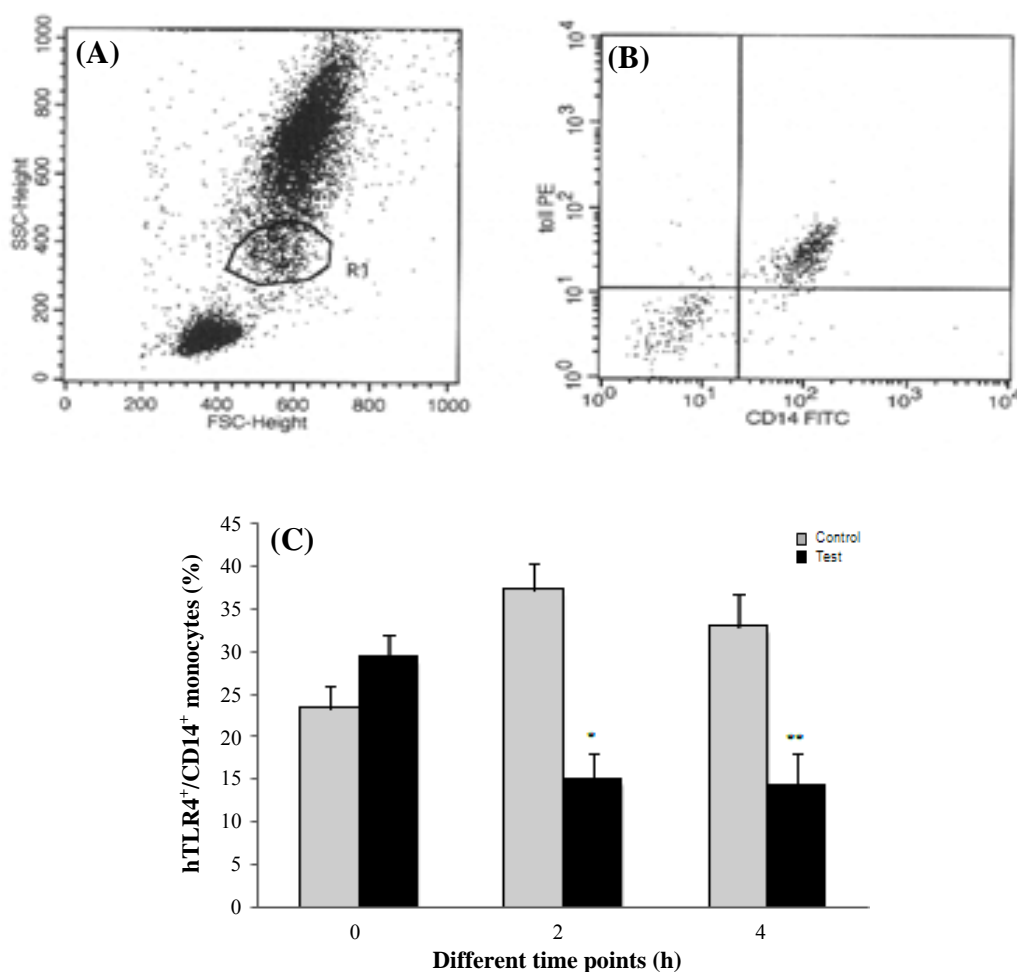


Fig. 1. Monocyte expression of hTLR4 in the study subjects. (A) Gating monocyte population. (B) Representative dot plot showing hTLR4⁺/CD14⁺ monocytes. Frequency of hTLR4⁺/CD14⁺ on circulating monocytes at different time points (%) (C). Data are shown as mean \pm SD. Cells (n = 10,000) were analyzed using flowcytometry in controls and patients before PCI (0h) and 2 hour (2 h) and 4 hour (4 h) after PCI, * $P < 0.01$ vs. control group at 2h; ** $P < 0.01$ vs. control group at 4h.

inflammation are used prognostically for patients with acute coronary syndromes. It is well established that monocytes contribute to all steps of atherosclerosis, from plaque formation to plaque instability and finally thrombosis [21, 22]. In concert with T cells, they secrete multiple cytokines and enzymes that contribute to plaque rupture. Significant data support the expression of TLR4 on the surface of monocytes not only in blood but also in arterial intima during atherogenesis [23, 24]. Fishbein *et al.* [25] demonstrated that in atherosclerotic lesions TLR4 is mainly expressed on monocytes and up-regulated by oxidized LDL, suggesting a link between lipids, inflammation, and atherosclerosis. A study showed that patients with acute coronary syndromes expressed enhanced TLR4 circulating monocytes compared to the patients with stable angina [6]. In addition, in an experimental study, inhibition of TLR4 caused a remarkable reduction in detrimental effects of

myocardial ischemia-reperfusion [26]. During injury, HSP-60 and HSP-70 may be released and trigger inflammatory response through activation of hTLR4 [8]. This finding was supported by Yang *et al.* [27] that confirmed a time-dependent expansion of hTLR4 monocytes in patients with ST-elevation myocardial infarction who received urokinase as a reperfusion therapy. They also showed that TNF- α serum levels were increased in a time-dependent manner after a successful reperfusion therapy. In contrast, Versteeg *et al.* [28] provided evidence that PCI negatively

Table 3. Serum levels of IL-1 β (pg/ml) at different time intervals

Group	Time intervals (h)		
	0	2	4
Control (n = 41)	14.8 \pm 1.8	30.1 \pm 4.0	18 \pm 2.4
Test (n = 30)	15.1 \pm 3.3	9.1 \pm 2.3*	8.5 \pm 1.4

* $P < 0.01$ vs. control group at 2 h

downregulated hTLR4⁺ monocytes in patients with chronic stable angina. However, its contribution to the inflammatory responses is an area of controversy. Inoue *et al.* [29] findings supported the PCI role in inflammation. Our previous study also proved that PCI positively regulated hTLR⁺/CD14⁺ monocytes and increased serum levels of pro-inflammatory cytokines [17], whereas opposite results were found in other studies [28, 30]. It is of note that most of investigations support the critical role of inflammation in restenosis even after implantation of drug-eluting stents. Along with multiple components of inflammation, cytokines play important roles in atherosclerosis. Ridker *et al.* [31] demonstrated that patients with higher levels of TNF- α were more prone to recurrent coronary events after myocardial infarction suggesting the role of cytokines in pathogenesis of myocardial infarction. On the other hand, IL-1 β induces the activation and proliferation of monocytes and stimulates further production of cytokines in atherogenesis [32]. Contrary to expectations, glucocorticoids administration has not been a successful treatment and did not improve clinical outcome. Hoeven *et al.* [33] reported that treatment with dexamethasone-eluting stents in patients with diabetes mellitus is linked with higher restenosis rate in patients without diabetes mellitus. In contrast, Kakio *et al.* [34] revealed that administration of hydrocortisone reduced the rate of in-stent restenosis. Taken together, it seems that current results are far from conclusive and are not uniformly consistent. Currently, it is not known with certainty that how hydrocortisone can influence expression patterns of hTLR4⁺/CD14⁺ monocytes and related downstream signaling. It should be further investigated whether it can impair protein or mRNA levels of hTLR4. It is noteworthy that statins have been suggested to suppress monocyte expression of hTLR4. However, no significant difference was seen among our studied patients in the use of medications. In conclusion, in the present study, we could demonstrate that hydrocortisone reduces monocyte expression of hTLR4 and pro-inflammatory cytokines. This finding may be applied in other inflammatory disorders in which hTLR4 are involved. Beneficial effects of glucocorticoids in reduction of coronary restenosis and possible association between reduction in hTLR4 expression and incidence of stent thrombosis or other coronary events should be adequately assessed. Furthermore, an effective and standard therapy to prevent stent thrombosis should be ascertained. Some limitations of our study should be acknowledged. Due to limited number of patients, our data should be confirmed in larger studies. It is worth noting that only a short-term effect of hydrocortisone on hTLR4 was assessed. Accordingly, more studies are needed to ascertain whether the effect of repeated doses of

hydrocortisone on hTLR4 expression persists at long-term follow-up.

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