EXPERIMENTAL STUDY

The therapeutic effects of thalidomide and etanercept on septic rats exposed to lipopolysaccharide

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ABSTRACT

BACKGROUND: The aim of this study was to evaluate the therapeutic effects of thalidomide and etanercept on lipopolysaccharide (LPS)-induced sepsis in a rat model.

METHODS: Thirty male Wistar Albino rats were divided into 5 groups: Control, LPS, LPS+Thalidomide, LPS+Etanercept, and LPS+Thalidomide+Etanercept. The control group was given a 1 mL intraperitoneal (i.p.) injection of 0.9% saline solution. For endotoxic treatment, the rats were injected with a single i.p. dose of LPS (Escherichia coli 0111:B4 (5 mg/kg). Thalidomide (0.5 mg/kg) and etanercept (1 mg/kg) were administered i.p. to the therapeutic groups. Hepatic tissue tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), intercellular adhesion molecule-1 (ICAM-1) and platelet-derived growth factor (PDGF) levels were determined by enzyme-linked immunosorbent assay and malondialdehyde (MDA) levels and total oxidant status (TOS) were measured using appropriate methods.

RESULTS: In vivo results exhibited elevated liver tissue TNF-α, IL-6, ICAM-1, PDGF, MDA, and TOS levels in the LPS-treated animals compared with the controls. The analysis of liver tissue supported the findings of biochemical alterations and indicated a therapeutic role for thalidomide and etanercept. Treatment of septic animals with these agents resulted in a remarkable decrease in the selected proinflammatory cytokines, angiogenic factors, and reactive oxygen parameters.

CONCLUSION: Restoration of cytokine balance and oxidant status to normal levels following treatment with selected therapeutic agents suggests that thalidomide and etanercept can help to avoid the potentially devastating effects of sepsis.

Keywords: Etanercept; lipopolysaccharide; sepsis; thalidomide.

INTRODUCTION

Sepsis is a very complex clinical state. It is described by the induction of a systemic inflammatory response due to infection.[1] This process causes tissue damage that may lead to multiple organ dysfunction like shock, with widespread morbidity and mortality.[2] The high mortality of sepsis indicates that the laboratory findings are not sufficient.

To limit tissue damage, pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) increase as part of the inflammatory response.[3] However, excess production of pro-inflammatory cytokines affects the normal immune response, and it causes a pathological condition. TNF-α has a major role in this process; therefore, it is considered as the primary mediator in the initial stage of inflammation.[4]

The IL-6 production is induced by bacterial endotoxins, IL-1, and TNF-α.[5] A high interrelation between mortality and IL-6 levels in patients with sepsis has been observed;[6] for this reason, IL-6 may be a good indicator of the severity of systemic bacterial infection.[7]

Recently, it has been directed toward by much research that the molecular basis of inflammation is the structure of cell surface receptors involved in cellular adhesion and signal transduction.[8] In addition to cytokines, enhanced expression

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of cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) has a significant function in the pathophysiology of sepsis.[9]

Platelet-derived growth factor (PDGF) has angiogenic effects, and it stimulates endothelial cell migration.[10] Although little is known about their regulation during the septic response, it promotes endothelial repair and angiogenesis.[11]

Severe oxidative stress results from oxidant-antioxidant imbalance in patients with sepsis, and it leads to oxidative modification of cellular macromolecules, structural tissue damage, and inducing cell death.[12] Malondialdehyde (MDA) measurement can be considered valuable for the indication of oxidative damage.[12] Moreover, the separate measurement of different oxidant molecules is impractical, and their oxidative effects are cumulative, so the total oxidant capacity of a sample may be measured by providing total oxidant status (TOS) values.[13]

Many in vivo models of sepsis have been used to evaluate various therapeutic agents. To this end, it was examined if the effects of thalidomide and etanercept on LPS-stimulated increased TNF-α, IL-6, ICAM, and PDGF levels. It was also aimed to examine whether there is an association between inflammatory mediators and oxidative stress parameters in sepsis.

MATERIALS AND METHODS

Animals

This novel study was conducted on 30 one-month-old male Wistar-albino rats, weighing 100–150g, purchased from the Firat University Experimental Animal Center Elazig, Turkey. The rats were housed in plastic cages with a controlled temperature of 22°C, humidity of 50%–55%, and a 12h light/dark cycle. All the animals had free access to food (regular rodent diet) and tap water. Animal procedures for this study were approved by Clinical Ethics Committee and the Animal Care Committee of Firat University.

Experimental Design

The rats were divided into five groups (n=6 per group) as follows:

Group 1 (Control): Rats received only 1 mL intraperitoneal (i.p.) injection of 0.9% saline solution.

Group 2 (LPS): Rats were administered a single i.p. injection of LPS (E. coli, 0111: B4; Sigma-Aldrich, St Louis, USA) at a dose of 5 mg/kg body weight. LPS was freshly prepared in saline.

Group 3 (LPS+Thalidomide): Thalidomide was given i.p. within its therapeutic anti-inflammatory dose (ED50 for rats, 0.5 mg/kg body weight).

Group 4 (LPS+Etanercept): Etanercept was administered i.p (ED50 for rats, 1 mg/kg body weight) to the animals.

Group 5 (LPS+Thalidomide+Etanercept): Thalidomide and etanercept were administered within their therapeutic anti-inflammatory dose as mentioned above.

All treatment agents were administered after being suspended in fresh DMSO 10%, and treatment of thalidomide and etanercept alone or in combination was performed at half an hour later LPS administration.

The entire application process took 6 h. After the experiment ended all rats were sacrificed under ketamine (75 mg/kg bw.t. Ketalar®, Pfizer Pharma GMBH, German) and xylazine (10 mg/kg body weight Alfazyne®, 2%, Alfasan International, 3440 AB, Woerden, Holland); and the hepatic specimens were taken.

Preparation of Tissue Homogenates

The hepatic tissues were weighed and homogenized in cold 0.01 M PBS buffer (1/10, w/v; pH:7.4) using automatic tissue homogenizer (Ultra TurraxType T25-B, IKA Labortechnic, Germany) at 3000 rpm for 10 min at 4°C. The homogenates were centrifuged at 5000 × g for 10 min at 4°C, and the supernatants were placed into the sterile tubes and stored at −80°C until analysis. Concentration of the protein was determined according to the procedure described by Lowry et al.[14]

Measurement of Biochemical Parameters

The TNF-α, IL-6 (SunRed, Shanghai, both of them), ICAM-1 (Boster Biological Technology, Pleasanton, CA), and PDGF (Elabscience, Wuhan, China) levels in liver supernatant were determined by rat enzyme-linked immunosorbent assay (ELISA) kits, according to manufacturer's recommendations. The optical density of each well was measured at 450 nm using an ELX 800 ELISA reader. The concentrations were calculated based on standard curves.

Levels of the TOS (Rel Assay Diagnostics, Gaziantep, Turkey) were determined by autoanalyzer (Siemens Advia 2400 Chemistry System, Siemens, Tokyo, Japan). The results were given in mmol/g protein.

The MDA measurements were evaluated using high-performance liquid chromatography (HPLC) system. The proteins in serum samples were precipitated with acid, and they were then centrifuged at 4500 × g for 5 min. The resulting supernatant by adding thiobarbituric acid (TBA) reagent was incubated for 45 min in a water bath at 90°C. The TBA-MDA product obtained after incubation was extracted with isobutanol. Measurements were performed by injecting 20 µl of the butanol phase into an HPLC system. 1,1,3,3-tetraethoxypropane was used as the MDA standard, and the results were given in nmol/g protein.

Statistical Analysis

The results are expressed as the mean±SD. Statistical significance was calculated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. All statistical analyses were performed with the SPSS 22.0 statistical software package for this purpose. P<0.05 was considered statistically significant.

RESULTS

The levels of tissue TNF-α, IL-6, ICAM-1, and PDGF were found statistically significantly higher in the sepsis group than the control group (for TNF-α p<0.05; for all the others p<0.001). At same time, treatment with both thalidomide and etanercept alone and in combination were found to statistically dramatically decrease the levels of TNF-α, IL-6, ICAM-1, and PDGF when compared to the sepsis group (p<0.05, for TNF-α and IL-6; p<0.001, for ICAM-1 and PDGF) (Table 1). Higher liver tissue TNF-α, IL-6, ICAM-1, and PDGF levels are associated with severe bacterial infection. These pro-inflammatory cytokines and angiogenic factors may be important in the endothelial dysregulation seen in sepsis. Therapeutic agents used in this study can help to avoid devastating effects of sepsis.

The levels of tissue MDA and TOS in the sepsis group were much higher than those in the control group (p<0.001, for both). The MDA levels were significantly lower in whole treatment groups than the sepsis group (p<0.05), but most significant decrease was observed in combined treatment group (p<0.001). At the same time, the levels of TOS also decreased in all treatment groups, and the most significant decrease was found in LPS+etanercept and LPS+thalidomide+etanercept group (p<0.001, both of them) (Table 2).

DISCUSSION

In this study using a rat model of LPS-induced sepsis, increased selected parameters levels in septic rats were found than those in the controls. In addition, this study suggests that thalidomide and etanercept might have clinical utility as a novel therapeutic approach for sepsis treatment.

Table 1. The mean tissue TNF-α, IL-6, ICAM-1, and PDGF levels in all experimental groups

Groups	TNF-α (ng/g prot)	IL-6 (pg/g prot)	ICAM-1 (ng/g prot)	PDGF (ng/g prot)
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Control	10.97±3.96	5187.72±1078.82	56.31±12.58	70.49±12.97
LPS	32.60±19.90*	1603563±6044.60**	193.97±75.97**	1044.99±475.72**
LPS+Thalidomide	11.85±6.06^{+}	7499.57±2594.29^{+++}	131.10±8.25^{++}	282.18±159.55^{+}
LPS+Etanercept	13.55±4.03^{++}	8379.30±2327.97^{+++}	127.72±66.39^{++}	392.46±324.18^{+}
LPS+Thalidomide+Etanercept	11.92±6.09^{+}	7615.96±2229.73^{+++}	115.09±36.43^{++}	229.75±95.37^{+}

*Compared with the control group; p=0.001. $^{+}$Compared with the LPS group; p=0.001. **Compared with the control group; p=0.000. $^{++}$Compared with the LPS group; p<0.005. $^{+++}$Compared with the LPS group; p=0.000.
TNF-α: Tumor necrosis factor; IL-6: Interleukin-6; ICAM-1: Intercellular adhesion molecule-1; PDGF: Platelet-derived growth factor. LPS: Lipopolysaccharide; SD: Standard deviation. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was carried out on the significance of p value. p values <0.05 were considered as statistically significant. Numerical variables were expressed as mean±standard deviation.

Table 2. The mean tissue MDA and TOS levels in all experimental groups

Groups	Malondialdehyde (nmol/gr prot)	Total oxidant status (mmol/gr prot)
	Mean±SD	Mean±SD
Control	21.66±5.84	0.20±0.23
LPS	65.66±33.89**	1.19±0.61**
LPS+Thalidomide	39.13±13.35^{++}	0.74±0.12^{++}
LPS+Etanercept	40.81±13.37^{++}	0.48±0.28^{+}
LPS+Thalidomide+Etanercept	25.24±8.67^{+}	0.29±0.28^{+++}

*Compared with the control group; p=0.001. $^{+}$Compared with the LPS group; p=0.001. **Compared with the control group; p=0.000. $^{++}$Compared with the LPS group; p<0.005. $^{+++}$Compared with LPS group; p=0.000.
MDA: Malondialdehyde; TOS: Total oxidant status; LPS: Lipopolysaccharide; SD: Standard deviation. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was carried out on the significance of p value. p values<0.05 were considered as statistically significant. Numerical variables expressed as mean±standard deviation.

The relationship between pathological conditions and TNF based on inflammatory processes has been investigated in our and many other studies. For instance, in endotoxin-derived animal and human sepsis models, TNF-α production is rapidly activated and can be measured in the plasma.[15,16] It was shown in a study that TNF-α could be a reliable indicator of severity of aggravation regardless of the etiologic factor of disease and as a prognostic factor.[17] Guo et al.[18] found that the TNF-α levels were highest in the early period after injection (30 min to 1 h) in a sepsis model elicited by a lipopolysaccharide injection. Furthermore, liver injury during endotoxemia emerges because of overproduction of many pro-inflammatory mediators such as TNF-α and IL-6, relative to all hepatic cell types.[19] In our study, hepatic TNF-α was also measured in place of plasma concentrations because localized cytokine concentrations were more prevalent in hepatic leucocyte uptake in the liver. In fact, hepatic, but not circulating, TNF-α modified gene expression was found in the liver through paracrine function.[20] Therefore, measurement of TNF-α in plasma may not be sufficient to determine concentrations in the liver.[21]

Administration of anti-TNF-α antibody inhibits TNF-α and protects animals from the lethal effects caused by lipopolysaccharide, gram-positive, and gram-negative bacteria challenge.[22] Suppression of TNF-α by etanercept may have a major function in limiting inflammation in the event of an excessive immune response.[23] Thalidomide is known to inhibit TNF-α through expedited degradation of messenger RNA.[24] Our study was coherent with above studies that found increased TNF-α levels. At the same time, it was observed that both etanercept and thalidomide treatment was convenient in decreasing TNF-α levels. It was suggested that this decrease was due to anti-TNF-α feature of both treatment agents.

Previous studies have found a high correlation between IL-6 levels and mortality in patients with sepsis;[25] for this reason, IL-6 may be a good indicator of the severity of systemic bacterial infection.[7] In comparison to C-reactive protein or procalcitonin (PCT), IL-6 levels peak 2 h after initiation of the inflammatory cascade. Depending on this rapid increase, IL-6 was presented as an indicator of early sepsis in emergency units.[26] It was shown in a multicenter study that IL-6 may predict survival at 28 days after the onset of sepsis.[27] Martin et al.[28] showed that compared to nonseptic newborns, serum IL-6, IL-8, and TNF-α levels were higher in septic. It can be said that among the cytokines produced in the liver during inflammation, TNF-α and IL-6 are important because of their numerous biological effects both in the liver and elsewhere.[21] Hence, in our study, we measured liver tissue levels of IL-6 and achieved similar results, considering that upregulation of TNF-α resulted in activated and increased IL-6 levels. The reduction in IL-6 production suggests that the therapeutic agents used may have positive effect on inflammation process.

ICAM-1 is one of the most important adhesion molecules that determine the adhesion and migration of neutrophils to target organs. However, overexpression of ICAM-1 on endothelial cells from non-specific organs may be a leading cause of organ dysfunction caused by sepsis.[29] In many studies conducted to investigate the direct role of ICAM-1 in sepsis, animals with anti-ICAM-1 antibody or gene deficiency were used, but inconsistent results were found.[8,30] Some studies have shown that blockade of ICAM-1 reduces survival in septic animals,[31] others suggest a beneficial role for ICAM-1 deficiency.[29,31] van Griensven et al.[30] used a cecal ligation and puncture sepsis model in their study, and they argued that the different model might be the reason of the contradictory results because some early studies use different models of bacterial injection. In our study, it was thought that the reason of the increased ICAM-1 expression can be stimulated by pro-inflammatory cytokines. It was known that etanercept and thalidomide could also modulate biological responses that are regulated or induced by TNF, including expression of adhesion molecules responsible for leukocyte migration like ICAM-1. So, it was possible to reduce the ICAM-1 levels for this reason.

It is unclear whether PDGF is a valuable agent in acute crucial statuses involving severe trauma, shock, and sepsis. In a study, PDGF and ICAM-1 levels were observed upregulated in septic neonates compared with those in normal. In our study, it was found not only several cytokines but also growth factors have contributed to the pathogenesis of sepsis.[32] It was concluded that PDGF is released as an immediate response to infection, and it may contribute to the systemic inflammatory response; it was considered to be the reason for increased PDGF levels in the sepsis group.

Oxidative stress has also a major function in the pathophysiology of sepsis. Studies about human and animal sepsis models have confirmed the presence of severe oxidative stress in patients with sepsis. For instance, an increased level of lipid peroxidation products including MDA was observed due to oxidative stress especially in adults with severe sepsis in non-survivors.[33] The findings further strengthened the function of oxidative stress as a mechanism of organ malfunction and thus became a potential therapeutic target in sepsis. It was assumed in a study that lipid peroxidation resulted in production of reactive oxygen species that played a significant function in pathogenesis of multiple organ malfunction and septic shock associated with neonatal sepsis. Lorente and colleagues have reported increased levels of MDA in severe sepsis compared to those in healthy controls.[34] Furthermore, increased MDA levels were also determined in the hepatic and renal tissues of septic rats.[35] Considering that the levels of TOS and MDA we evaluated as the oxidative parameter in the sepsis group were higher than those in the other groups, oxidant-antioxidant balance seems to progress toward oxidants in inflammation. After using treatment agents, the decreased MDA and TOS levels

were probably the result of inactivation by lipid peroxyl radicals and their breakdown products.

In conclusion, our study may establish an example for future work aimed at better understanding sepsis in experimental rat models. We conclude that higher liver tissue TNF-α, IL-6, ICAM-1, and PDGF levels may be associated with severe bacterial infection. These pro-inflammatory cytokines and angiogenic factors may be important in the endothelial dysregulation seen in sepsis. Moreover, therapeutic agents used in this study can help to avoid devastating effects of sepsis, but there is a need for more comprehensive experimental studies for usage in the treatment of sepsis.

Conflict of interest: None declared.

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DENEYSEL ÇALIŞMA - *ÖZET*

Talidomid ve etanersept'in lipopolisakkarite maruz bırakılan septik sıçanlarda terapötik etkileri

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AMAÇ: Bu çalışmada lipopolisakkaritle (LPS) indüklenmiş sıçan sepsis modelinde talidomid ve etanersept'in terapötik etkilerini değerlendirmeyi amaçladık.

GEREÇ VE YÖNTEM: Otuz adet erkek Wistar Albino sıçan; Kontrol, LPS, LPS+Talidomid, LPS+Etanersept ve LPS+Talidomid+Etanersept olmak üzere beş gruba ayrıldı. Kontrol grubuna 1 mL %0.9'luk salin solüsyonu intraperitoneal (i.p.) olarak verildi. Endotoksik tedavi için sıçanlara LPS (Escherichia coli 0111: B4 (5 mg/kg), tek doz) i.p. olarak enjekte edildi. Terapötik grupları oluşturmak için talidomid (0.5 mg/kg) ve etanersept (1 mg/kg) i.p. olarak uygulandı. Hepatik doku tümör nekroz faktör-α (TNF-α), interlökin-6 (IL-6), hücreler arası adezyon molekülü-1 (ICAM-1) ve platelet-türevli büyüme faktörü (PDGF) düzeyleri ELISA yöntemiyle, malondialdehid (MDA) düzeyi ile toplam oksidan kapasitesi (TOS) uygun yöntemler kullanılarak ölçüldü.

BULGULAR: In vivo sonuçlar, kontrol ile karşılaştırıldığında LPS uygulanmış hayvanlarda karaciğer TNF-α, IL-6, ICAM-1, PDGF, MDA ve TOS düzeylerinin arttığını gösterdi. Karaciğer dokularının analiziyle, biyokimyasal değişiklikler desteklendi ve talidomid ve etanersept'in terapötik rolünü kanıtladı. Septik hayvanların bu tedavi ajanlarıyla tedavi edilmesiyle seçilen proenflamatuvar sitokinler, anjiyogenik faktörler ve reaktif oksijen parametrelerinde kayda değer bir düşüş gözlendi.

TARTIŞMA: Seçilen terapötik ajanların tedavisini takiben sitokin dengesinin ve oksidan durumunun normale döndürülmesi, talidomid ve etanersept'in sepsisin yıkıcı etkilerinden kaçınmaya yardımcı olabileceğini düşündürmektedir.

Anahtar sözcükler: Etanersept; lipopolisakkarit; sepsis; talidomid.

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