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Markers of endothelial cell activation and immune activation are increased in patients with severe leptospirosis and associated with disease severity

Marco Goeijenbier [a,*], M. Hussein Gasem [b,f], Joost C.M. Meijers [c,d], Rudy A. Hartskeerl [e], Ahmed Ahmed [e], Marga G.A. Goris [e], Bambang Isbandrio [f], Simone S. Schuller [g], Albert D.M.E. Osterhaus [a,h], Byron E.E. Martina [a,h], Eric C.M. van Gorp [a], Jarlath E. Nally [i], Jiri F.P. Wagenaar [a,e]

[a] *Department of Viroscience, Erasmus MC, Rotterdam, The Netherlands*
[b] *Department of Medicine, Dr. Kariadi Hospital, Faculty of Medicine, Diponegoro University, Semarang, Indonesia*
[c] *Department of Experimental Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands*
[d] *Department of Plasma Proteins, Sanquin Research, Amsterdam, The Netherlands*
[e] *WHO/FAO/OIE and National Leptospirosis Reference Centre, KIT Biomedical Research, Amsterdam, The Netherlands*
[f] *National Reference Laboratory for Leptospirosis, Dr. Kariadi Hospital, Semarang, Indonesia*
[g] *Vetsuisse Faculty, University of Bern, Bern, Switzerland*
[h] *ARTEMIS One Health Research Institute, Utrecht, The Netherlands*
[i] *Bacterial Diseases of Livestock Research Unit, National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Ames, IA, United States*

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Summary *Objectives:* Previous studies concluded that haemorrhage is one of the most accurate prognostic factors of mortality in leptospirosis. Therefore, endothelial cell activation was investigated in relation to disease severity in severe leptospirosis.
Methods: Prospective cohort study of severe leptospirosis patients. Plasma levels of sE-selectin and Von Willebrand factor (VWF) were determined. Consequently, an *in vitro*

* Corresponding author. P.O. Box 2040, 3000 CA Rotterdam, The Netherlands. Tel.: +31 10 7030310.
E-mail address: m.goeijenbier@erasmusmc.nl (M. Goeijenbier).

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sFas ligand;
sIL-2 receptor

endothelial cell model was used to assess endothelial activation after exposure to virulent *Leptospira*. Finally, immune activation, as a potential contributing factor to endothelial cell activation, was determined by soluble IL2-receptor (sIL-2r) and soluble Fas-ligand (sFasL) levels.
Results: Plasma levels of sE-selectin and VWF strongly increased in patients compared to healthy controls. Furthermore, sE-selectin was significantly elevated (203 ng/ml vs. 157 ng/ml, $p < 0.05$) in survivors compared to non-survivors. Endothelial cells exposed to virulent *Leptospira* showed increased VWF expression. E-selectin and ICAM-1 expression did not change. Immunohistochemistry revealed the presence of intracellular *Leptospira* and qPCR suggested replication. *In vivo* analysis showed that increased levels of sFasL and sIL-2r were both strongly associated with mortality. Furthermore sIL-2r levels were increased in patients that developed bleeding and significantly correlated to duration of hospital stay.
Discussion: Markers of endothelial activation and immune activation were associated with disease severity in leptospirosis patients.

Introduction

Leptospirosis is an infectious disease of global importance.[1] The disease is caused by spirochaetes that are spread by the urine of infected animals. Mucous membranes, small cuts and abraded skin are the usual points of entry. Although the clinical course varies widely, mortality rates of up to 52% of patients hospitalized with severe leptospirosis have been reported while in patient subgroups with leptospirosis pulmonary haemorrhage syndrome even rates up to 85% have been reported in literature.[2–4] The more common mild form of leptospirosis is characterized by non-specific symptoms such as: acute fever, headache, chills, myalgia and conjunctival suffusion.[5] In most severe forms, the clinical picture of leptospirosis may encompass jaundice, renal failure and haemorrhaging of skin, mucous membranes and/or lungs. Post mortem pathological findings confirm widespread haemorrhaging and endothelial cell dysfunction resulting in generalized oedema.[6,7]

Clinical data from patients with severe leptospirosis show significant activation of coagulation, which in many cases is followed by (often fatal) haemorrhage.[8,9] Although the pathophysiology of the haemorrhagic diathesis in leptospirosis remains unclear, bleeding could very well be the result of endothelial cell dysfunction, since the endothelium is the key player in regulation of haemostasis.[10,11] Under physiological conditions the vascular endothelium inhibits coagulation, prevents platelet aggregation and due to low levels of expressed adhesion molecules, it precludes adherence and migration of leukocytes. Injury or activation of endothelial cells in response to pathogens or to inflammatory cytokines can cause bleeding due to the loss of integrity of the blood vessel resulting in consumptive coagulopathy and/or vascular leakage.[12] Thus far, *in vitro* work showed the ability of *Leptospira* to transmigrate through endothelial cell monolayers.[13] Furthermore, an increase in adhesion molecules on the surface of human umbilical vein endothelial cells (HUVEC) was measured, when cells were incubated with *Escherichia coli* expressing leptospiral outer membrane proteins or with a recombinant leptospiral lipoprotein.[14,15]

However, the exact role for endothelial cell activation, or source of damage, in the pathogenesis of leptospirosis remains unclear. In particular, it is not known whether haemorrhage is a direct function from exposure to the pathogen, or indirectly via host response factors.[16] The aim of this work was to investigate endothelial cell activation in relation to bleeding in patients suffering from severe leptospirosis using a combined *in vivo*, *in vitro*, *in vivo* approach. To do so, we first determined the state of the endothelium in patients with severe leptospirosis, using soluble E-selectin (sE-selectin), a marker of activity expressed exclusively on the surface of endothelial cells[17] and Von Willebrand factor (VWF), also a sensitive marker of endothelial cell activation. We then evaluated the interaction between virulent *Leptospira* and endothelial cells *in vitro* using HUVEC. Combined, these results led us to hypothesize that T-cells play a more prominent role in the pathogenesis of severe leptospirosis. This hypothesis was tested in patients with severe leptospirosis by measuring the T-cell activation marker soluble IL2-receptor (sIL2-r) and the immune mediated cell damage marker soluble Fas Ligand (sFasL).

Materials and methods

Patients and controls

Consecutive patients with severe leptospirosis were included from February 2005 till September 2006 at the Dr. Kariadi hospital, Semarang, Indonesia. Severe leptospirosis was defined as hospitalized patients with a high clinical suspicion of leptospirosis, presenting with at least one of the following symptoms or signs: jaundice, renal failure, thrombocytopaenia and/or bleeding and a positive LeptoTek Dri-Dot assay (Biomérieux), confirmed by microscopic agglutination test (MAT). We defined bleeding as the spontaneous occurrence of: petechiae, ecchymosis, epistaxis, gum bleeding, haematuria, melaena, haematemesis and/or haemoptysis. Sepsis was defined using standard sepsis criteria.[18] This was a static classification scored on admission; progression from e.g. sepsis to severe sepsis within days was not taken into account. After written informed consent was given, blood samples were taken on admission and during follow up at day 1, 2, 7 and 14. Citrated blood was centrifuged immediately and plasma aliquots were stored at −70 °C until further analyses. As controls, 20 healthy Indonesian (Javanese) volunteers (no fever, no complaints at time of blood withdrawal) were tested. The medical ethics committee the faculty of