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Full Length Article

Toll-like receptor 9 gene expression in the post-thrombotic syndrome, residual thrombosis and recurrent deep venous thrombosis: A case-control study

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ABSTRACT

Objective: Animal models suggest that toll-like receptor 9 (TLR9) promotes thrombus resolution after acute deep venous thrombosis (DVT). We hypothesized that TLR9 expression is lower in patients with post-thrombotic syndrome (PTS) and investigated the role of TLR9 in residual thrombosis (RT) and recurrence.
Methods: Patients with a history of DVT with PTS (cases, n = 30) and without PTS after minimal 24 months follow-up (controls, n = 30) were selected. Healthy individuals (HI, n = 29) without DVT were included as reference. TLR9 mRNA expression in leukocytes was determined by qPCR and normalized to the housekeeping succinate dehydrogenase subunit A gene using the ΔCt method. Sub analyses were performed to explore the TLR9 expression in patients with and without RT and multiple DVT episodes.
Results: The median TLR9 expression was 0.45 (interquartile range 0.31 to 0.93), 0.39 (0.25 to 0.69) and 0.62 (0.32 to 0.75) in cases, controls and HI respectively ($p = 0.61$). The median TLR9 expression was 0.39 (0.26 to 0.51) in patients with RT compared to 0.55 (0.30 to 0.86, $p = 0.13$) in those without. The median TLR9 expression was significantly lower in patients who had one DVT compared to patients with recurrent DVT, 0.37 (0.23 to 0.63) versus 0.55 (0.43 to 0.96) respectively ($p < 0.01$).
Conclusion: No significant difference in TLR9 expression was found between cases, controls and HI. However TLR9 expression seems lower in individuals with DVT and RT, albeit not significant. Interestingly, TLR9 might play a role in recurrent DVT, as the TLR9 expression was significantly higher in patients with recurrent DVT.

1. Introduction

Post-thrombotic syndrome (PTS) is a common complication of deep vein thrombosis (DVT), which occurs in 20–50% of patients after deep vein thrombosis [1–4]. The clinical presentation may vary from minor signs including skin discoloration, venous ectasia, discomfort and swelling, to severe manifestations such as chronic pain, intractable edema or leg ulcers impairing daily activities [5,6]. Due to its high prevalence, severity and chronicity, PTS has a significant impact on quality of life and is associated with considerable socio-economic consequences for both the patient and the health care system [4,6].

The pathogenesis of PTS is not yet fully understood. It has been proposed that the symptoms of PTS are caused by the end-organ manifestation of venous hypertension, as a result of several processes such as tissue remodeling, impaired thrombus resolution and continued inflammation [7–9]. Besides Virchow's triad, there are strong indications that inflammation also plays a role in the pathogenesis of DVT [10]. During an inflammatory process multiple procoagulant mechanisms are stimulated, whereas anticoagulant mechanisms are inhibited. Although immune-mediated inflammation plays a role in the thrombotic process, little is known about the role of innate immunity.

Toll-like receptor 9 (TLR9) is a member of the toll-like receptor family mainly expressed on the membrane surface of endosomes and lysosomes of leukocytes [11]. TLR9-mediated recognition of (viral or bacterial) unmethylated CpG dinucleotides triggers a T-lymphocyte helper 1 (Th1) immune response directed against the intracellular

Abbreviations: ACTB, beta-actin; BMI, body mass index; DVT, deep venous thrombosis; HI, healthy individuals; IL-6, interleukin-6; IL-8, interleukin-6; IQR, interquartile range; RT, residual thrombosis; SDHA, succinate dehydrogenase subunit A; TLR9, toll-like receptor 9; Th1, T-lymphocyte helper 1.

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pathogen. TLR9 has also been involved in thrombus formation and resolution [10,12,13]. In animal studies, deletion of TLR9 was associated with significantly increased thrombus size, decreased neovascularization, fibrosis and thrombus resolution. This may be due to the fact that deletion of TLR9 lowers the levels of Th1 cytokines (INFα, IL-1α, IL-2), whereas inflammation is needed in the process of thrombus resolution. Therefore decreased cytokine levels could account for impaired thrombus resolution [13]. In a prospective study in humans, TLR9 expression was down-regulated in patients with a DVT at the time of diagnosis compared to healthy controls [10].

So far, the association between TLR9 and PTS in humans has not been investigated. As deletion or low levels of TLR9 are associated with larger thrombi and less thrombus resolution, we hypothesized that TLR9 expression is lower in patients with PTS. Furthermore we investigated the role of TLR9 in residual thrombosis (RT) and recurrent DVT.

2. Materials and methods

2.1. Patients and study design

A case-control study was performed, including 30 patients with a history of DVT who developed PTS (cases) and 30 patients with a history of DVT without PTS (controls). Thirty healthy individuals (HI) without a history of venous thromboembolism were invited to participate as a reference population. The cases and controls were selected from a cohort of patients that was followed prospectively after acute DVT. Patients were recruited from the Maastricht University Medical Centre or the Flevohospital in Almere, the Netherlands. Patients with a history of DVT and PTS development (Villalta ≥ 5) were defined as cases [5,14]. Patients with a history of DVT and without PTS development (Villalta ≤ 4) after a minimal follow-up of 2 years after DVT, were defined as controls. Subjects or patients with known venous insufficiency were excluded from the study because of possible interference with the endpoints. Cases, controls, and HI were similar for gender, age and body mass index.

RT was determined with ultrasonography 3–12 months after treatment with anticoagulation.

The medical ethical committee of the Maastricht University Medical Centre approved the study and all patients gave written informed consent.

2.2. Measurement of TLR9 gene expression

Venous blood was drawn from all subjects in EDTA polypropylene tubes for plasma. The EDTA tubes were centrifuged for 5 min at 2500 g (3790 rpm, room temperature). One milliliter of buffy coat was separated and 10 ml RNA/DNA Stabilization Reagent for Blood/Bone Marrow (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) was added to the buffy coat and mixed well. The samples were stored at −20 °C until analysis.

Total RNA was purified from the preserved buffy coats with the High Pure RNA isolation kit (Roche Diagnostics). After quantification at the NanoDrop, total RNA was subjected to reverse transcription with random primers at 37 °C for 120 min (High Capacity cDNA Reverse-Transcription kit, Life Technologies, Bleiswijk, the Netherlands). TLR9 mRNA expression in leukocytes was determined by qPCR on a LightCycler 480 (Roche Diagnostics) using validated TLR9-specific primers and hybridization probes (RealTime-ready assay, Roche Diagnostics) according to the manufacturer's instructions. Similar RealTime-ready assays were used to quantify the mRNA expression of the housekeeping genes mitochondrial succinate dehydrogenase subunit A (*SDHA*) and beta-actin (*ACTB*) [15]. TLR9 expression was normalized to the expression of SHDA and ACTB using the ΔC_t method. Relative expression was calculated as $2^{[-((C_t \text{ of TLR9}) - (C_t \text{ of reference gene}))]}$, C_t being the threshold cycle.

2.3. Statistical analyses

SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The non-parametric Kruskal-Wallis test was performed to test for differences in TLR9 expression levels between cases, controls and HI. Post-hoc Mann-Whitney *U* test was performed on significant ($p < 0.05$) Kruskal-Wallis test results. Sub analyses with the Mann-Whitney *U* test were performed to compare the TLR9 expression in patients with and without RT and in patients with one and multiple DVT episodes. TLR9 expression levels were described as medians and interquartile ranges (IQR). Multivariate regression analyses were performed to determine whether the use of statins, aspirin and anticoagulation affects the TLR9 expression. Spearman correlation was performed to test the association between the two housekeeping genes.

3. Results

The total study population consisted of 89 subjects; 30 cases, 30 controls and 29 HI were included. Ninety subjects were originally selected, but one HI was found ineligible because of the presence of venous insufficiency. The median age was 64 years (IQR 46 to 76) for cases, 67 years (IQR 58 to 76) for controls and 61 years (IQR 59 to 68) for the HI (Table 1). Blood collection was performed at a median follow-up of 85 months (IQR 58 to 122) after the first DVT in the cases and 86 months (IQR 50 to 99) after the first DVT in the controls (Table 1). Median Villalta score was 7 (IQR 6 to 9) for the cases and 2 (IQR 1 to 3) for the controls (Table 1). Use of aspirin was similar in cases, controls and HI ($p = 0.91$). Use of statins seemed higher in cases and controls, but this was not statistically significant ($p = 0.22$). Use of oral anticoagulants was significantly higher in cases as compared to controls and HI ($p < 0.01$).

3.1. TLR9 expression in cases, controls and HI

In line with data on record [15], ACTB mRNA (median C_t 15.5) was more abundant than SHDA mRNA (median C_t 24.5) in leukocyte total RNA, but the mRNA expression of these housekeeping genes was not influenced by the disease status (case, control or HI). The median C_t of TLR9 mRNA over the whole population was 25.3.

The median TLR9 expression relative to SHDA was 0.45 (IQR 0.31 to 0.93) in cases, 0.39 (IQR 0.25 to 0.69) in controls and 0.62 (IQR 0.32 to 0.75) in HI ($p = 0.61$, Fig. 1). Multivariate regression analysis did not show an effect of statins, aspirin or anticoagulant use on the expression of TLR9. The correlation (Spearman's coefficient) between SHDA-normalized and ACTB-normalized TLR9 mRNA expression levels in the

Table 1
Baseline characteristics.

	Cases (n = 30)	Controls (n = 30)	HI (n = 29)
Age, median (IQR)	64 (46–76)	67 (58–76)	61 (59–68)
Male, no (%)	15 (50%)	14 (47%)	15 (52%)
BMI, median (IQR)	28 (25–32)	25 (24–31)	26 (24–29)
Villalta score[a], median (IQR)	7 (6–9)	2 (1–3)	
Recurrent DVT	14 (47%)	9 (30%)	
Follow-up after first DVT, median months (IQR)	85 (58–122)	86 (50–99)	
Follow-up after most recent DVT, median months (IQR)	51 (31–73)	64 (41–89)	
Oral anticoagulant use	16	7	2
Acenocoumarol	13	7	1
Phenprocoumon	3	0	1
Statin use	11	10	5
Aspirin use	4	5	5

Abbreviations: HI, healthy individuals; IQR, Interquartile range; BMI, body mass index; DVT, deep venous thrombosis.
[a] Average of 1–4 measurements of Villalta score.