Targeting the vessel wall in cardiovascular prevention
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Chapter 5

Tumor Necrosis Factor-α inhibition protects against endotoxin-induced endothelial glycocalyx perturbation


* Both authors contributed equally to this manuscript

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ABSTRACT

Objective: Inflammatory stimuli profoundly increase the vulnerability of the vessel wall to atherogenesis. The glycocalyx, a layer of glycosaminoglycans and proteoglycans covering the endothelium, has recently emerged as an orchestrator of vascular homeostasis. In the present study, we investigated whether endotoxin-induced inflammatory reactions lead to a decrease of endothelial glycocalyx thickness in humans and whether Tumour Necrosis Factor-α (TNFα) plays a role in this process.

Design, subjects and intervention: Healthy male volunteers received low-dose endotoxin (1 ng/kg) intravenously, with (n = 8) or without (n = 13) pre-treatment with the soluble TNFα receptor etanercept. Endothelial glycocalyx thickness and related parameters were determined after endotoxin challenge.

Results: Endotoxin resulted in a profound reduction in microvascular glycocalyx thickness (from 0.60 ± 0.1 to 0.30 ± 0.1 μm, p < 0.01). Concomitantly, plasma levels of the principal glycocalyx constituent hyaluronan (62 ± 18 to 85 ± 24 ng/mL, p < 0.05), monocyte activation and coagulation activation (F1+2: 0.3 ± 0.1 to 2.8 ± 1.5 nmol/L, p < 0.05) increased. Inhibition of TNFα by etanercept attenuated loss of microvascular glycocalyx thickness (0.54 ± 0.1 to 0.35 ± 0.1 μm, p < 0.05). Changes in hyaluronan (58 ± 13 to 46 ± 10 ng/mL, p < 0.05) and coagulation activation (F1+2: 0.3 ± 0.1 to 2.1 ± 0.9 nmol/L, p < 0.05) were also attenuated.

Conclusions: These data suggest that inflammatory activity, in part mediated by TNFα, leads to perturbation of the endothelial glycocalyx in humans. This may contribute to the vascular vulnerability induced by inflammation.

Keywords: endothelial glycocalyx, endotoxin, TNFα, hyaluronan

Abbreviations: CRP, C-reactive protein; OPS, orthogonal polarization spectroscopy; TNFα, tumor necrosis factor-α
INTRODUCTION

Patients with chronic inflammatory disorders, such as rheumatoid arthritis and Crohn's disease, often suffer from accelerated atherogenesis (1-3). Besides that, elevated levels of endotoxin in the human bloodstream have been associated with an increased risk for atherosclerosis (4). Moreover, repeated administration of endotoxin has been shown to directly stimulate atherosclerotic lesion formation in experimental animal models (5). Even single inflammatory challenges, such as vaccination, infusion of C-reactive protein (CRP) or endotoxin administration have been associated with endothelial dysfunction in humans (6-8). Collectively, these data suggest that inflammatory stimuli increase the vulnerability of the vessel wall to atherogenic stimuli. The exact pathways contributing to the increased vulnerability, characterized by endothelial dysfunction, increased vascular permeability and increased leukocyte and platelet aggregation, have not been fully elucidated (9).

Recently, the endothelial glycocalyx has been put forward as an orchestrator of vascular homeostasis (10). This intraluminal layer, mainly consisting of heparan sulphate and hyaluronan, is instrumental for regulating vascular permeability (11, 12). Perturbation of the glycocalyx is also accompanied by increased leukocyte and platelet adhesion in experimental models (13, 14). Reconstitution of the glycocalyx, on the other hand, fully restores the protective properties of the vessel wall (11, 13). Tumour Necrosis Factor-α (TNFα) is one of the pivotal factors that have been shown to disrupt endothelial glycocalyx in an experimental model (15). This was closely correlated with increased vascular permeability as well as endothelial dysfunction. In humans, we recently showed that hyperglycaemia is characterized by glycocalyx perturbation, coinciding with endothelial dysfunction and coagulation activation (16).

We hypothesized that acute inflammatory stimuli adversely affect the endothelial glycocalyx in humans, thus augmenting vascular vulnerability. Therefore, we evaluated the effect of a standardized inflammatory challenge (endotoxin 1 ng/kg) on endothelial glycocalyx thickness, as well as on endothelial function, coagulation and inflammatory markers. To specifically address the role of TNFα, experiments were performed with or without pre-treatment with the soluble TNFα receptor etanercept.

MATERIALS AND METHODS

Study design

Twentyone healthy Caucasian male volunteers were studied (Table 1). The study was approved by the Institutional Review Board of the Academic Medical Centre, Amsterdam and written informed consent was obtained from all volunteers. Participants had no history of
cardiovascular disease, did not smoke, did not use any medication and were free from any febrile illness in the month preceding the study. Medical history, physical examination, routine laboratory examination, electrocardiogram and chest X-ray were normal. All experiments were performed after an overnight fast. A baseline measurement comprising microvascular (sublingual) glycocalyx thickness and biochemistry was performed in all subjects. Five days later, subjects were allocated to intramuscular injection of either saline (n = 13) or etanercept (n = 8; Enbrel® 50 mg, Wyeth, USA). After 48 hours, after an overnight fast, study participants were admitted to the research unit and a catheter was inserted in an antecubital vein of each arm. Blood was drawn for baseline measurements and hereafter subjects received a bolus infusion of 1 ng/kg body weight of endotoxin (Escherichia coli lipopolysaccharide, catalog number 1235503, lot G2B274; United States Pharmacopeial Convention Inc, Rockville, Md) in the antecubital vein of the contralateral arm. The incidence, time and severity of clinical symptoms associated with endotoxemia were recorded as previously published (17). Vital signs, including blood pressure, heart rate and body temperature, were measured at regular intervals at t = 0.5, 1, 3 and 4 hours after endotoxin challenge. The next morning, 24 hours after endotoxin infusion, study participants returned after an overnight fast for final blood withdrawal. Blood was collected in EDTA, citrate, and heparin anticoagulated aliquots, as

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Data are presented as mean ± SD.
well as serum tubes, which were kept on ice and centrifuged at 1600g for 15 minutes at 4°C. Plasma and serum were snap-frozen, and stored at -80°C until analysis.

**Estimation of microvascular glyocalyx**

Microvascular glyocalyx thickness was measured approximately 4 hours after endotoxin infusion, at the same time of day as the baseline measurement. Thickness of the endothelial glyocalyx in individual capillaries was estimated using orthogonal polarization spectroscopy (OPS) imaging of the sublingual microcirculation (18). The width of flowing erythrocytes was measured in individual capillaries before and immediately after the passage of a leukocyte through the capillary (19, 20, 21). This method is based on the linear theory model (22, 23, 24). In short, endothelial glyocalyx limits the proximity of erythrocytes to capillary endothelial cells (19). In contrast, leukocytes, which are much more rigid, compress the capillary endothelial glyocalyx during their passage through the capillary lumen, thus allowing a transient ‘widening’ of the erythrocytes following the leukocyte passage (23). Hence, the change in erythrocyte column diameter divided by two (referred to as delta erythrocyte width/2) is related to the dimension of the microvascular glyocalyx, that is transiently compressed by the passing leukocytes (20, 23).

The OPS procedure is non-invasive, painless and takes approximately 15 minutes. Measurements of the central sublingual area were performed with a handheld OPS camera (Cytometrics, Philadelphia, PA, USA). Pressure on the tissue was avoided to ensure normal flow. Images were collected with a 5× objective providing a 325× magnification and were sized 720 × 576 pixels. The frame rate was 25 per second. All frames were recorded on Sony DSR-20P digital video recorder and transferred to a computer using Windows Movie Maker. Analysis of the images was performed with Image-Pro Plus by a single image analyst, who was blinded for the clinical details of the participants. Capillaries with a diameter between 3 and 7 μm were selected for analysis. The anatomical capillary diameter and the width of the flowing erythrocyte column were measured using digital callipers prior to and after spontaneous capillary leukocyte passage (20). Per participant, glyocalyx dimension was determined in at least 5 capillaries. The mean of these results was calculated and used in further analyses. In addition, the number of capillaries per field was counted as indication of capillary density as previously described (25).

**Blood sampling and laboratory methods**

Leukocyte counts as well as subfractions were determined in whole blood with standardized flow cytometric analysis. Plasma CRP levels were measured with a commercially available assay (Roche, Switzerland). Plasma soluble TNFα receptor type 2 (sTNFR2) levels were measured using an enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc, Minneapolis, MN, USA), as a marker of effective etanercept administration (26). Plasma IL-6 levels, a cytokine
induced by TNFα activity, were measured using Cytometric Bead Array technique (R&D systems, Minneapolis, MN, USA). Prothrombin activation fragment F1+2 (Dade Behring, Marburg, Germany) was measured by ELISA to estimate thrombin generation. D-dimer levels were measured as a reflection of fibrin formation and subsequent endogenous fibrinolysis with an automated quantitative latex-particle immunoassay (Biomerieux, Durham, NC, USA). Quantitative plasma hyaluronan was measured by ELISA (Echelon Biosciences, Salt Lake City, UT, USA), which measures total amount (including low and high molecular weight) hyaluronan. Heparan sulphate was measured after serum pre-treatment with Actinase E (Sigma, St. Louis, MO, USA) by ELISA (Seikagaku Corporation, Tokyo, Japan). Total plasma hyaluronidase activity was determined with a previously published assay with minor modifications (27). In short, CovaLink plates (Nunc, Wiesbaden, Germany) were coated with biotinylated hyaluronan (0.2 mg/mL, HyluMed® Sterile IUO Sodium hyaluronate, Genzyme Corp, Cambridge, MA, USA). Plasma samples were diluted 800x and added to the plates for 2.5 hours at 37°C at pH 3.7. Bovine hyaluronidase (Sigma, St. Louis, MO, USA) was used for the standard curve. The remaining amount of hyaluronan was determined by binding of avidin-biotin complex (Vectastain, Vector Laboratories, Burlingame, CA, USA), followed by addition of o-phenylenediamine (OPD) and 30% H₂O₂. Plates were measured at OD 492 nm.

Monocyte flow cytometry procedure

Whole blood samples were collected in pyrogen-free lithium-heparin tubes and erythrocytes were lysed. For flow cytometric analysis, remaining cells were incubated in FACS buffer mixed with antibody. All reagents were titrated to obtain optimal results as recommended by the manufacturers. Cell surface staining was performed with Fluorescein Isothiocyanate (FITC) labelled mouse anti-human CD14 (IgG2a), anti-human Allophycocyanin (APC) labelled CD18 (IgG1) and Phycoerythrin (PE) labelled anti-human CD11b (IgG1) and CD62L (IgG1) (R&D Systems, San Jose, CA, USA). Appropriate isotype control antibodies were used to correct for non-specific antibody binding. After staining, the cells were washed, fixed in 4% paraformaldehyde, and analyzed by flow cytometry and at least 100,000 cells were counted per sample. Data were analyzed with CellQuest software.

Statistical analysis

All values are provided as means ± SD. Differences in baseline characteristics between the endotoxin-saline and the endotoxin-etanercept group were analyzed by two-tailed unpaired Student’s t test, since there were no indications for abnormal distribution of the data. Changes within treatment groups were analyzed by two-tailed paired Student’s T-test. Changes between treatment groups were analyzed by two-way analysis of covariance (interaction treatment and time). P < 0.05 was considered to represent a statistically significant difference.
RESULTS

Clinical responses to endotoxin infusion with or without etanercept pre-treatment

Prior to endotoxin infusion, no differences in clinical characteristics were observed between the saline and the etanercept group (Table 1). Infusion of endotoxin was well tolerated and no serious adverse effects were encountered. Endotoxin infusion caused characteristic clinical symptoms, such as chills, headache, myalgia and nausea. These symptoms were transient in both groups, but occurred more frequently and more intensive in the saline group compared to the etanercept group (data not shown). sTNFR2 levels were significantly increased after etanercept pre-treatment (from 1.8 ± 0.2 to 520 ± 34 ng/mL, p < 0.0001), indicating that etanercept was effectively administered. Plasma levels remained elevated 4 hours after endotoxin infusion in the etanercept group (646 ± 83 ng/mL), whereas sTNFR2 plasma levels were only slightly affected in the saline group (from 2.1 ± 0.3 to 5.2 ± 0.5 ng/mL 4 hours after endotoxin, p < 0.01). Blood pressure, heart rate and body temperature significantly changed 4 hours after endotoxin infusion in the saline group (systolic blood pressure: from 127 ± 11 to 119 ± 5 mmHg, ns; diastolic blood pressure: from 67 ± 8 to 51 ± 9 mmHg, p < 0.01; heart rate: from 59 ± 6 to 82 ± 5, p < 0.01; and body temperature: from 36.6 ± 0.5 to 38.3 ± 0.4 ºC, p < 0.01 compared to baseline for all parameters). Etanercept attenuated these changes (systolic blood pressure: from 125 ± 7 to 123 ± 10 mmHg, ns; diastolic blood pressure: from 69 ± 6 to 67 ± 6 mm Hg, ns; heart rate: from 62 ± 4 to 69 ± 3, ns; and body temperature: from 36.6 ± 0.5 to 37.3 ± 0.5 ºC, ns compared to baseline for all parameters).

Etanercept attenuated endotoxin-induced glycocalyx perturbation

Endotoxin infusion led to a reduction in microvascular glycocalyx thickness in the saline group (from 0.60 ± 0.1 to 0.30 ± 0.1 μm, p < 0.01; see Figure 1). Loss of glycocalyx resulted in increased capillary blood filling as reflected by an increased width of the capillary erythrocyte column (from 4.2 ± 0.1 to 4.9 ± 0.1 μm, p < 0.05) prior to leukocyte passage. No apparent changes were detected in the anatomic capillary diameters (from 5.4 ± 0.2 to 5.5 ± 0.1 μm, ns). Etanercept limited decreases in microvascular glycocalyx to 35% (from 0.54 ± 0.1 to 0.35 ± 0.1 μm, p < 0.05). Capillary density significantly decreased upon endotoxin challenge (from 60 ± 18 to 44 ± 16 per field, p < 0.01) with similar changes in the etanercept pre-treatment group (from 59 ± 7 to 43 ± 15 per field, p < 0.01).

Changes in glycocalyx components upon endotoxin infusion

Plasma hyaluronan levels, a marker of glycocalyx shedding, rose significantly within first hour after endotoxin infusion in the saline group (from 62 ± 18 to 85 ± 24 ng/mL, p < 0.05), whereas etanercept reduced endotoxin-induced shedding (from 58 ± 13 to 46 ± 10 ng/mL, p < 0.05) (Figure 2a). Plasma hyaluronidase activity was significantly decreased 4 hours after endotoxin infusion in the saline group (-56 ± 20% compared to baseline, p < 0.01), whereas
Hyaluronidase activity was not affected in the etanercept group (-8 ± 14% compared to baseline, ns; Figure 2b). Notably, heparan sulphate plasma levels did not significantly change during the 4 hours after endotoxin challenge with either pre-treatment (saline group: from 5.3 ± 1.1 to 5.5 ± 1.2 μg/mL versus etanercept group: from 5.4 ± 1.3 to 5.1 ± 1.0 μg/mL, ns compared to baseline). However, 24 hours after endotoxin infusion plasma heparan sulphate levels increased, especially in the saline group (11.2 ± 2.1 μg/mL versus etanercept group 7.4 ± 1.5 μg/mL, p < 0.01).

**Etanercept treatment reduced TNFα-induced inflammatory and coagulation responses**

Inflammatory markers rose in the saline group after endotoxin challenge (IL-6: from 4.2 ± 6.3 to 678 ± 427 pg/mL and CRP: from 0.5 ± 0.4 to 26.7 ± 7.6 mg/L, p < 0.01 compared to baseline, see Figure 3a and b). Etanercept significantly reduced this increase (IL-6: from 4.6 ± 3.1 to 127 ± 98 pg/mL, p < 0.05 compared to baseline and CRP: from 0.6 ± 0.4 to 16.0 ± 3.4 mg/L, p < 0.01 compared to baseline). In parallel, 4 hours after endotoxin, the number of circulating leukocytes was doubled in both treatment groups compared to baseline.

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**Figure 1. Endothelial glycocalyx thickness**

Endothelial glycocalyx thickness was determined before and after endotoxin challenge without (left bars) or with etanercept pre-treatment (right bars). Data are presented as means ± SD (* p < 0.05, # p < 0.01).
Figure 2a. Plasma hyaluronan levels
Plasma hyaluronan levels in human volunteers challenged with endotoxin without (black bars) or with etanercept pre-treatment (open bars). Data are presented as means ± SD (* p < 0.05 compared to baseline; # p < 0.05 between groups).

Figure 2b. Plasma hyaluronidase activity
Plasma hyaluronidase activity in human volunteers challenged with endotoxin without (dots) or with etanercept pre-treatment (diamonds). Data are presented as means ± SD (* p < 0.05 vs. baseline, # p < 0.05 between groups).
Markers of inflammation were assessed by plasma IL-6 levels (Figure 3a, without (dots) or with (diamonds) etanercept pre-treatment) and plasma CRP plasma levels (Figure 3b, without (black bars) and with (white bars) etanercept pre-treatment) during endotoxin challenge in human volunteers. Data are presented as means ± SD (* p < 0.05 vs. baseline, † p < 0.01 vs. baseline, # p < 0.05 between groups).
With respect to leukocyte differentiation after endotoxin infusion, a significant drop in monocyte count of 76 ± 29% was observed in the saline group at 4 hours (p < 0.01 compared to baseline), whereas etanercept was associated with a less profound reduction of 51 ± 32% (p < 0.05 compared to baseline). There was no difference between CD14+ monocyte counts.
between the two groups (27 ± 10 vs. 26 ± 13%, ns). However, the CD11b⁺/CD18⁺ monocyte count was higher after saline compared to etanercept (median 0.82 ± 0.2 versus 0.65 ± 0.1, p < 0.01). Finally the percentage of CD62L⁻ (L-selectin) expressing monocytes was decreased maximally 4 hours after endotoxin in the saline group whereas in the etanercept group an increase was observed (-8 ± 37% vs. + 66 ± 42%, p < 0.05).

In parallel, markers of endotoxin-induced thrombin generation and subsequent fibrinolysis in saline group were significantly increased, starting 3 hours after endotoxin challenge (F1⁺₂: from 0.3 ± 0.1 to 2.8 ± 1.5 nmol/L and D-dimer: from 0.2 ± 0.1 to 0.4 ± 0.1 mg/L, p < 0.05 compared to baseline; Figure 4a and b), whereas etanercept significantly reduced this increase (F1⁺₂: from 0.3 ± 0.1 to 2.1 ± 0.9 nmol/L, and D-dimer: from 0.2 ± 0.1 to 0.3 ± 0.1 mg/L, p < 0.05).

DISCUSSION

In the present study we show that a low-dose endotoxin challenge leads to loss of endothelial glycocalyx and shedding of the glycocalyx constituent hyaluronan into the plasma compartment. These changes were accompanied by reduction in perfused capillary density, increased monocyte activation and thrombin generation. Conversely, we show that inhibition of TNFα activity with etanercept attenuates the reduction in endothelial glycocalyx perturbation, abolishes shedding of glycocalyx constituents, and reduces coagulation activation. These findings imply a profound effect of inflammatory activation on endothelial glycocalyx, which may contribute to loss of vascular protection.

Effect of endotoxin on glycocalyx thickness and capillary density

The role of the endothelial glycocalyx as a target of damage and, conversely, as a potential structure providing protection against atherogenic stimuli, is emerging rapidly. Although the techniques to evaluate the endothelial glycocalyx in humans are relatively new, our data provide solid information to extend these emerging hypotheses (20). In the present study, we observed a large decrease in endothelial glycocalyx thickness. This corroborates with previously published data showing that TNFα directly damages the glycocalyx in a hamster model (15). Moreover, shedding of endothelial glycocalyx upon pro-inflammatory stimuli has been described several times (14, 28, 29).

Besides loss of endothelial glycocalyx, we found a decrease in capillary density. OPS images revealed that there was a 50% reduction in sublingual endothelial glycocalyx thickness in about 70% of the capillaries that remained perfused after endotoxin challenge. These findings are in line with a study by Cabrales et al. (30). This group showed that degradation of the glycocalyx leads to capillary perfusion impairments, a reduction functional capillary density
and an increased erythrocyte flux in the remainder of perfused capillaries in a hamster model. We speculate that this decrease in glycocalyx thickness could lead to reduction in microvascular resistance and account for the observed reduction in diastolic blood pressure.

**Effect of endotoxin on glycocalyx components**

Endotoxin challenge results in a large reduction in microvascular glycocalyx thickness. The concomitant rapid increase in circulating plasma hyaluronan levels implies increased shedding as a cause for loss of glycocalyx. In line, previous studies demonstrate shedding of endothelial glycosaminoglycans upon inflammation (31, 32). However, hyaluronan concentrations only rose modestly compared to the large reduction in glycocalyx. This may be explained by rapid uptake of excess hyaluronan in the liver (33). Hyaluronan contributes to vascular permeability barrier properties, as selective removal of hyaluronan with hyaluronidase has been shown to be accompanied by a clear increase in vascular permeability for macromolecules (11, 12). Increased circulating hyaluronan levels in conjunction with a dramatic increase in vascular permeability are also found in septic patients (32). Of note, plasma hyaluronidase activity was decreased, rather than increased. This could pertain to the release of endogenous hyaluronidase inhibitors, which are increased during inflammatory reactions (34).

**Effect on coagulation and inflammatory markers**

Our study confirms previously published data that endotoxin activates the coagulation system as well as monocytes (35, 36). This may point towards a role for increased reactive oxygen species (ROS) generation in endotoxin-induced glycocalyx damage (29, 37). Interactions between monocytes and endotoxin involve CD14, a glycoprotein present on mononuclear and polymorphonuclear leukocytes. CD14 acts as a high-affinity receptor for complexes of endotoxin and endotoxin-binding protein. Endotoxin binding to CD14 and TLR-4 activates both endothelium as well as monocytes, resulting amongst others in increased TNFα and hyaluronan release (36, 38). The ensuing loss of endothelial glycocalyx could facilitate binding of monocytes to activated endothelium, illustrated by increased CD11b+/CD18+ expression in the monocytic fraction. This is line with data from animal studies showing that perturbation of endothelial glycocalyx indeed results in increased leukocyte adhesion (13, 14, 15, 28).

**Study limitations**

Etanercept is a dimeric fusion protein between the recombinant form of the human p75 TNFα receptor 2 and the Fc fragment of human IgG1 and can therefore be measured in the assay for native sTNFR2 (26). Prior studies have shown an increase in circulating TNFα in response to TNFα inhibition with recombinant soluble TNFα receptors (35). We also measured an increase in TNFα in the etanercept group (data not shown). This likely relates to sequestration of etanercept with TNFα in the circulation, as both free TNFα and that bound to the sTNFR2 are measured in the TNFα assay. We measured sTNFR2 plasma levels for treatment efficacy in
combination with CRP and IL-6 plasma levels as a secondary cytokine following TNFα, as bio-assays for TNFα activity are not widely available. As shown, both CRP and IL-6 were indeed attenuated upon etanercept pre-treatment. Second, the technique to estimate endothelial glycocalyx in humans is indirect and should be interpreted with caution.

Clinical implications
Coagulation and inflammation are entangled with endothelial dysfunction (39). Both atherosclerosis and sepsis are associated with inflammatory activation, increased vascular permeability and subsequent vascular damage. Animal studies indicate that the endothelial glycocalyx is a crucial intravascular compartment which modulates vascular permeability and serves as a barrier attenuating leukocyte and platelet adhesion. Our data point towards a potential role of endothelial glycocalyx in the protection of the vessel wall. This study provides the foundation for further studies of the endothelial glycocalyx in humans to discern its exact role in the mentioned maladies and to determine whether preventing glycocalyx perturbation can attenuate the adverse effects of inflammatory stimuli on the endothelium.

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DISCLOSURES

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