The Dynamics of Gene Expression in Human Lung Microvascular Endothelial Cells after Stimulation with Inflammatory Cytokines

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Received September 20, 2004 Accepted March 21, 2005 On-line available April 26, 2005

Summary

Vascular endothelium plays an essential role in the pathogenesis of vasoocclusion. The changes in the endothelial cell function can be triggered by changes in gene expression caused by interaction with cytokines and blood cells. Using cDNA arrays, we have recently reported complex patterns of gene expression after stimulation of endothelial cells with TNF α and IL-1 β . Better understanding of the time course of gene expression changes, their concentration dependence and reversibility after withdrawal of the offending cytokine is essential for successful prevention and therapy of vasoocclusion. Here we present a detailed study of the concentration dependence and time course of gene expression in endothelial cells after their exposure to TNF α and IL-1 β . We focus on the adhesion molecules (VCAM-1, ICAM-1, E-selectin) and cytokines (IL-6, GCP-2, MCP-1) that are likely to contribute to vasoocclusion. We report differences in the time course and intensity of their expression and in their response to TNF α and IL-1 β stimulation. We demonstrate that expression of the studied genes is upregulated by low TNF α concentrations that better reflect the TNF α levels detected in the plasma of patients developing vasoocclusion. These results help to understand the changes in the endothelium and to design rational prevention and therapy of vasoocclusion.

Key words

Vasoocclusion • TNF α • IL-1 β • Microvascular endothelial cells • Gene expression • cDNA array

Introduction

The vascular endothelium comprises the inner layer of all vessels and serves as a barrier between blood and various tissues. It receives and transfers biochemical and biophysical information in both directions, thereby actively participating in the basic physiological processes. It plays an important role in angiogenesis (Hanahan and Folkman 1996), maintains a balance between its anticoagulant and potential procoagulant capabilities (Esmon 1987, Rodgers 1988), helps to control vascular tone by releasing either vasodilatory or vasoconstricting factors, (Brenner *et al.* 1989) and plays an important role in the inflammatory process (Pober and Cotran 1990). Most importantly, endothelial cells (EC) provide a responsive surface that is modulated by an extraordinary

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ISSN 0862-8408 Fax +420 241 062 164 http://www.biomed.cas.cz/physiolres array of biological modifiers, among others by inflammatory cytokines such as TNF α and IL-1 β (Pober 1988, Favaloro 1993). These factors modulate surfacedependent functions of EC, which can underlie the onset of vascular disorders such as the vasoocclusive processes in diabetes mellitus, atherosclerosis, malaria or sickle cell anemia (Cines *et al.* 1998)

Proinflammatory cytokines TNF α and IL-1 β induce changes in gene expression in the EC. Among others (Natarajan et al. 1996), TNFa and IL-1B increase the expression of adhesion molecules ICAM-1, E-selectin and VCAM-1 (Haraldsen et al. 1996), and chemokines and cytokines responsible for inflammatory reaction, such as IL-8, MCP-1 (Goebeler et al. 1997), MIP-2 (Liu et al. 2000), IL-6 (Hettmannsperger et al. 1992), RANTES (Shukaliak and Dorovini-Zis 2000), GCP-2 (Beck et al. 1999) and CX3CL1 (Garcia et al. 2000). Since TNFa and IL-1 β are secreted in high levels in many pathophysiological situations, deregulation of these genes significantly contribute vasoocclusive can to complications.

Another significant contributor to endothelial pathobiology is the heterogeneity of EC, in particular the difference between cells derived from the large vessels and from the microvasculature. Although the majority of previous in vitro studies of EC activation have used human umbilical vein endothelial cells (HUVEC), most vasoocclusive events are linked to microvascular endothelial cells (HMVEC). Multiple differences between HUVEC and HMVEC have been reported. Among others, HMVEC differ from HUVEC in the expression of cell surface markers such as glycoprotein CD36 (Salcedo et al. 2000, Swerlick et al. 1992) and chemokine receptors (Salcedo et al. 2000). The responses of these two types of endothelial cells to stimulation with cytokines also differ, as was shown in the studies of the release of CXC-chemokines (Beck et al. 1999, Haraldsen et al. 1996). These data document the importance of using relevant endothelial cell culture system for the in vitro assessment of EC activation.

We have recently published gene expression profiles of HMVEC stimulated with the inflammatory cytokines TNF α and IL-1 β (Zachlederova and Jarolim 2003). We used an *in vitro* model of endothelial cell activation and studied expression profiles of more than 2000 genes using commercially available cDNA microarrays. We reported complex changes in gene expression in endothelial cells including markedly increased expression of genes that have not been previously associated with EC stimulation. Description of these novel genes can lead to new therapeutic approaches to vasoocclusive crises in sickle cell anemia and other disorders of microvasculature. However, in order to establish optimal timing and dosage of therapeutic interventions, detailed understanding of the time and concentration dependence of the gene expression is necessary. In the current study, we therefore provide an evaluation of the concentration and time-dependences of gene expression for selected adhesion molecules, chemokines and cytokines.

Methods

Endothelial cell culture

Human microvascular endothelial cells derived from lung (HMVEC-L) (Clonetics, San Diego, CA) were grown in the EGM2-MV medium supplemented with 5 % Fetal Bovine Serum (Clonetics) at 37 °C in a 5 % CO_2 humidified air incubator. All experiments were performed between passages 5 and 7 on the first day of 100 % confluence.

Co-incubation of HMVEC-L with inflammatory cytokines

TNF α and IL-1 β (R&D systems, Minneapolis, MN) were added to the fresh medium and incubated with HMVEC-L at concentrations and for times described in the results.

RNA isolation

RNA was isolated using Trizol Reagent (Life Technologies, Rockville, MD) according to the Trizol Reagent protocol. RNA was treated with 1 U RQ1 RNase-free DNase (Promega, Madison, WI) and cleaned with the RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA quality was checked by electrophoresis in a 1.2 % agarose gel in 0.5x TBE buffer and its concentration was estimated spectrophotometrically.

Gene expression profiling

The Atlas Human cDNA Expression Arrays 1.2 were purchased from Clontech, Palo Alto, CA. Equal amounts of radioactively labeled cDNA probes prepared from total RNA from different samples were hybridized in parallel to the Atlas Array membranes according to manufacturer's instructions. The hybridized membranes were exposed to a phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA) at room temperature for several hours. The phosphorimaging screen was scanned using PhosphorImager SI (Molecular Dynamics). The gene expression was quantified using the Atlas Image software (Clontech). Normalization of the corresponding arrays was based either on the average expression of all genes or on the expression of the housekeeping genes, whichever was more appropriate. All HMVEC-L treatments were repeated twice. A minimum twofold change in the gene expression in two independent experiments was considered significant.

Quantification of mRNA

cDNA was prepared from total RNA by the Super Script II reverse transcriptase (Life Technologies) and random hexamers (Promega) and PCR-amplified by 0.4 µM gene-specific primers (sequences obtained from Clontech) in a reaction mix containing 1x PCR buffer, 1.5 mM MgCl₂ (Promega), 0.2 mM each dNTPs, and 1 U Taq DNA polymerase (Promega). The following program was used: 94 °C for 1 min followed by a specific number of cycles of 94 °C for 30 s, 60 °C for 30 s, and 74 °C for 30 s. The number of cycles was determined for each studied gene separately. The reaction was stopped after 18, 20, 22, 24, 26, 28 and 30 cycles for each gene and each mRNA sample. After the electrophoresis, the gel was photographed using the Alpha Imager 2000 (Alpha Innotech Corp., San Leandro, CA) and the bands were quantified using the ImageQuant software (Molecular Dynamics). A logarithmic dependence of band intensity on the number of cycles was calculated. The number of amplification cycles for which the logarithmic dependence was linear was determined for every examined mRNA. The intensity of the PCR band for each specific gene was normalized to the intensity of the corresponding 18S rRNA band from the same sample. Primers for 18S rRNA were used in a 3:7 dilution with the 18S competimers (QuantumRNA 18S Internal Standards, Ambion, Austin, TX).

Results

Gene expression profiles of HMVEC-L incubated with TNF α or IL-1 β

In a recently published study, we compared the gene expression profiles of HMVEC-L incubated for 20 h in the presence and absence of 5 ng/ml TNF α or 50 pg/ml IL-1 β using the Atlas Human cDNA Expression Arrays. (Zachlederová and Jarolím 2003). In this follow-up study, we focused on the genes encoding ICAM-1, VCAM-1, E-selectin, IL-6, MCP-1, and GCP-2. Although the

overall EC responses to both stimuli were similar, we observed several significant differences in the expression of the studied genes. The VCAM-1 gene responded exclusively to TNF α . Expression of other genes was stimulated by both cytokines, but the ICAM-1, GCP-2 and E-selectin upregulation was significantly higher after TNF α treatment when compared to co-incubation with IL-1 β . Changes in expression of these genes are summarized in Table 1. We also confirmed the Expression Array results using semi-quantitative RT-PCR. Fig. 1 shows an increase in mRNA expression relative to the mRNA levels in unstimulated HMVEC-L.

Table 1. Upregulation of gene expression in HMVEC-L incubated with 5 ng/ml TNF α and with 50 pg/ml IL-1 β . Array position describes coordinates of a gene on Array 1.2. The increase in expression is described by + (2-5 fold), ++ (5-10 fold) or +++ (more than 10 fold). Blank cells correspond to less than twofold change in gene expression.

Gene	Expro TNFa	ession IL-1β
VCAM-1 (vascular cell adhesion molecule 1)	+++	
E-selectin (endothelial leukocyte adhesion molecule 1)	+++	+++
ICAM-1 (intercellular adhesion molecule 1)	+++	++
MCP-1 (monocyte chemotactic protein 1)	+++	+++
GCP-2 (granulocyte chemotactic protein 2)	+++	+
IL-6 (interleukin 6)	+	+

Concentration dependence of gene expression

HMVEC-L were incubated in the presence of TNF α concentrations ranging from 0.01 to 5 ng/ml. Expression of selected genes was assessed by semiquantitative PCR. We observed at least a twofold increase in expression of the ICAM-1, MCP-1 and GCP-2 genes, even at the lowest tested concentration of TNF α (0.01 ng/ml). Expression of VCAM-1 and E-selectin genes was significantly increased at concentration of 0.1 ng/ml TNF α . Gene for IL-6 was upregulated only slightly even at the TNF α concentration of 5 ng/ml, while no increase in its expression was detectable at lower concentrations (Fig. 2).



Fig. 2. Concentration dependence of gene expression. HMVEC-L were incubated in the presence of TNF α in the range from 0.01 to 5 ng/ml for 20 h. Gene expression was measured by RT-PCR. Ratio of the mRNA content in stimulated cells to the mRNA content in control HMVEC-L.

Time dependence of gene expression

HMVEC-L were incubated in the presence of 5 ng/ml TNF α or 50 pg/ml IL-1 β for 1.5, 4, and 20 h. Expression of selected genes was measured by semiquantitative PCR. The studied genes can be divided into three groups according to the time course of their expression: early responders whose expression after stimulation with cytokines increases rapidly during the first 90 min and then remains constant or decreases (Fig. 3a); intermediate responders whose expression increases within the first 4 h of cytokine presence (Fig. 3b); late responders whose expression keeps increasing up to the 20-h time point (Fig. 3c).

Genes for ICAM-1 and IL-6 responded differently to the TNF α and IL-1 β stimulation. While the ICAM-1 gene followed the early response pattern after IL-1 β stimulation, longer incubation was required for the full response to TNF α . On the other hand, the response of the IL-6 gene to IL-1 β was slower than the response to TNF α . Expression of the VCAM-1 gene followed the intermediate gene response after both types of stimulation but while it remained high even at the 20-h time point after TNF α treatment, it decreased rapidly to baseline after IL-1 β stimulation. While E-selectin belongs to the early genes in both types stimulation, MCP-1 and GCP-2 belong to the late genes. An increase in expression of GCP-2 in the first 4 h of incubation with TNF α is not detectable.

Pulse-chase experiments

To study the long-term effect of TNF α stimulation, we incubated HMVEC-L in the presence of TNF α (5 ng/ml) for one hour and then washed the cytokine out and continued incubation in the medium without TNF α . We measured gene expression at the 1-h, 4-h, and 20-h time intervals. Figure 4 compares the time course of gene expression in samples with continuous presence of cytokine and with TNF α washed out after one hour.

Fig. 1. Confirmation of the gene expression changes by RT-PCR. RT-PCR was performed for RNA isolated from control HMVEC-L or HMVEC-L treated with TNF α (5 ng/ml) or IL-1 β (50 pg/ml) for 20 h. Intensity of the PCR band for each specific gene was normalized to the intensity of the corresponding rRNA 18S band from the same sample. Ratio of the mRNA content in stimulated cells to the mRNA content in the control HMVEC-L is plotted in the y-axis. In each of the six bar graphs, the first lane corresponds to gene expression in control HMVEC-L normalized to 1, and lanes two and three to relative gene expression in HMVEC-L treated with TNF α and IL-1 β , respectively.



Fig. 3. Time dependence of gene expression. HMVEC-L were incubated in the presence of $TNF\alpha$ (5 ng/ml) or IL-1 β (50 pg/ml) for 1.5, 4 and 20 h. Gene expression was measured by RT-PCR. Ratio of the mRNA content in stimulated cells to the mRNA content in the control HMVEC-L is plotted on the y-axis. a) Early responders whose expression after stimulation with cytokines increases rapidly during the first 90 min and then remains constant or decreases; b) Intermediate responders whose expression increases within the first 4 h of cytokine presence; c) Late responders whose expression keeps increasing up to the 20-h time point.

An increase of ICAM-1 gene expression triggered by TNF α in the first hour continued even in the absence of the cytokine and peaked at 4 h. The expression level at this time point was similar to the expression in samples with the continuous presence of TNF α . After 4 h, the ICAM-1 expression decreased but it remained above baseline even after 20 h. A similar course can be seen for expression of the E-selectin gene, albeit the levels of E-selectin after removing TNF α are considerably lower than in its continued presence. Increase in IL-6 expression was rapid in the first hour of TNF α presence but decreased to baseline in the absence of the cytokine. Expression of MCP-1 slightly increased after 1-h incubation with TNF α , but it remained constant even after 20 h in the absence of TNF α . VCAM-1 expression increased moderately after 1-h incubation with TNF α , but it decreased back to baseline after TNF α removal. One-hour presence of TNF α was insufficient for inducting the GCP-2 gene.

Discussion

The objective of this study was to complement our previous work on gene expression profiles of endothelial cells stimulated with proinflammatory cytokines (Zachlederova and Jarolim 2003) by studying the dynamics of expression of selected genes. We continued using the primary culture of human



Fig. 4. Pulse-chase study of gene expression. HMVEC-L were incubated in the presence of TNF α (5 ng/ml) for 1 hour, then the cytokine was washed and incubation continued in the medium without TNF α . Gene expression was measured by RT-PCR after 1, 4 and 20 hours of incubation. Ratio of the mRNA content in stimulated cells to the mRNA content in the control HMVEC-L is plotted on the abscissa. Each chart compares the gene expression in the continuous presence of TNF α (dotted line) with the pulse-chase experiment (full line).

microvascular endothelial cells (HMVEC) as a physiologically more relevant *in vitro* model of the pathogenesis of microvascular changes. We focused on the expression of genes encoding ICAM-1, VCAM-1, E-selectin, IL-6, MCP-1 and GCP-2, all of which are likely to play an important role in the pathogenesis of vasoocclusion. ICAM-1, VCAM-1 and E-selectin are adhesive molecules that are essential for leukocyte trafficking in infection or inflammation. IL-6, MCP-1 and GCP-2 are cytokines that contribute to the inflammatory reaction.

Increased expression and elevated plasma levels of these molecules were detected in various pathological conditions including those with vasoocclusive complications such as diabetes mellitus (Gabazza *et al.* 1996, Kado *et al.* 1999, Lechleitner *et al.* 2000, Morii *et al.* 2003) atherosclerosis, (de Lemos *et al.* 2003, Signorelli *et al.* 2003) and sickle cell anemia (Duits *et al.* 1996, Makis *et al.* 2000, Solovey *et al.* 1997, Taylor *et al.* 1995). In several *in vitro* experiments, increased expression of these genes was triggered by TNF α and IL-1 β (Beck *et al.* 1999, Goebeler *et al.* 1997, Haraldsen *et al.* 1996, Hess *et al.* 1996, Hettmannsperger *et al.* 1992, Kanda *et al.* 1998, Wong and Dorovini-Zis 1995, Zachlederova and Jarolim 2003). While plasma concentrations of TNF α are increased in various pathological situations, their levels vary widely not only among different diseases but also among different

patients with the same disease. As an example, TNF α levels in plasma of patients with type-1 diabetes mellitus were increased to 19.3±7.5pg/ml in comparison with healthy controls (11.1±5.8 pg/ml) (Lechleitner *et al.* 2002). Patients with type-2 diabetes had plasma TNF α levels of 28.0±13.8 pg/ml in comparison to 16.2±9.6 pg/ml in non-diabetic controls. (Lechleitner *et al.* 2000) Median TNF α level was 26 pg/ml in patients with sepsis, ranging from 0 to 1000 pg/ml (Casey *et al.* 1993). In patients with newly diagnosed lymphoma, the TNF α levels oscillated between 5 and 380 pg/ml with the median of 20 pg/ml, while the TNF α levels in healthy controls were between 4 to 9 pg/ml with the median of 7 pg/ml (Salles *et al.* 1996).

Even when the plasma levels of TNF α are known, local concentrations of inflammatory cytokines in the endothelial microenvironment during vasoocclusive events may be different. These findings can complicate the interpretation of results obtained from *in vitro* experiments with defined TNF α concentrations. We therefore studied the dependence of gene expression on a wide range of TNF α concentrations. We found that most genes with increased expression after the 5 ng/ml TNF α treatment also responded to considerably lower concentrations of TNF α , often to the lowest TNF α concentration tested in this study (10 pg/ml). This result supports our assumption that our *in vitro* model of endothelial activation may correspond to a real *in vivo* situation.

Characterization of the kinetics of gene expression is important not only for understanding of the pathophysiology of vasoocclusion, but also for the design of potential therapeutic interventions. First, it is important to know how quickly the targeted genes respond to an increase in pro-inflammatory cytokines. If the mRNA levels increase with a significant delay, a therapeutic intervention aimed at blocking or removing the offending stimulus, such as application of an anti-TNF α antibody, plasma exchange, or similar effects may succeed. However, if the stimulus already triggered increased gene expression, its removal may not be warranted. Second, it is important to know how fast the gene expression can return to its original level after removal of the stimulus. Therapeutic removal of proinflammatory cytokines may succeed for genes whose expression requires continuous presence of the stimulating cytokine, even if their RNA

levels have already been increased by cytokine stimulation. On the other hand, removal of the stimulus may not be sufficient for downregulation of genes with stable mRNAs or genes regulated by positive feedback.

We therefore studied the time course of expression of selected genes not only in the presence of TNF α , but also in a pulse-chase experiment, in which we co-incubated HMVEC-L with TNF α for one hour, and subsequently removed $TNF\alpha$ and measured gene expression after 4 and 20 h. The RNA levels for several genes continued to rise or remained elevated even after the removal of the stimulus. In particular, ICAM-1 expression reached similar levels 3 h after TNF α removal and in its 4-h presence. Even though the ICAM-1 expression decreased 4 h after removal of the cytokine, it remained significantly elevated even after 20 h. Similarly, the expression of E-selectin and MCP-1 remained increased for extended periods of time after removing TNF α , albeit it was significantly lower than that in the presence of TNFa. These results demonstrate that for the above genes, a therapeutic intervention aimed at preventing vasoocclusion and based on the removal of TNF α or, possibly, other pro-inflammatory cytokines, may not succeed if ICAM-1, E-selectin and MCP-1 are the critical genes involved in the pathogenesis of vasoocclusion. In contrast, it may successfully prevent or reverse up-regulation of VCAM-1 or IL-6.

One obvious limitation of our approach is the well-known fact that the mRNA levels may not correspond to the resulting levels of corresponding proteins. Consequently, potential therapeutic interventions based on the results of this study would have to be validated by a study of protein expression. However, a detailed understanding of the concentration dependence and time course of expression of important adhesion molecules and chemokines in HMVEC after stimulation with proinflammatory cytokines can help to design therapeutic interventions that might prevent or decrease the severity of vasoocclusive events.

Acknowledgement

This work was supported by the Research Scholar Program of the Boston Comprehensive Sickle Center HL15157-30 and by the Grant CEZ MZ 237360001 of the Grant Agency of the Czech Ministry of Health.

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Reprint requests

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