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Original paper

EFFECTS OF CYTOKINE MIXTURES ON THE EXPRESSION OF ADHESION MOLECULES IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS IN AN *IN VITRO* MODEL OF INFLAMMATION

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Summary: As an essential early event in the activation of the immune system increased adherence of circulating neutrophils, lymphocytes and monocytes to the microvascular endothelium is observed. This situation is followed by migration of these cells through vessel walls and their accumulation at sites of tissue injury. This process is mediated by specific cell adhesion molecules being crucial to the generation of immune and inflammatory responses. In this report we demonstrate the effects of cytokine stimulation on endothelial adhesion molecules evoked by incubating HUVECs with two specific cytokine combinations both comprising IL-2, IL-6, IFN-γ and TNF-α, which have been selected because they are elevated in the blood during rejection and infection processes. Combination I additionally includes IL-8, which is released by activated monocytes and macrophages and is suggested to be an important angiogenic mediator stimulating the proliferation and migration of endothelial cells. On the other hand, combination II contains the two anti-inflammatory cytokines IL-4 and IL-10, which are predominantely synthesised by Th2 cells. While IL-4 demonstrates multiple stimulatory and regulatory effects, IL-10 plays a pivotal part in the regulation of immune responses. Both cytokines block the synthesis of cytokines, such as IL-1, TNF-α and IL-12, which are of regulatory importance at the beginning of inflammatory processes. These cytokine mixtures are placed in the centre of our studies in order to elucidate their influence on the cell surface expression of a number of adhesion molecules on HUVECs, when combined in multi-component incubation cocktails. The application of these cytokine combinations results in comparable effects significantly increasing the mean fluorescence intensity (MFI) of VCAM-1, slightly up-regulating ICAM-1 surface expression accompanied by the induction of E- and P-selectin expression. These adhesion molecules play pivotal parts in the process of leukocyte transmigration. The experiments reveal a strong up-regulation of these cell surface antigens under conditions mimicing inflammation. This is an essential finding stressing the importance of endothelial cells during the activation of the immune system.

Key words: endothelial cells, adhesion molecules, fluorescence activated cell scanning, cytokines, inflammation.

Introduction

The localisation of leukocytes plays a pivotal role in the different phases of an inflammatory process. Adhesion molecules are of prominent importance for the migration of leukocytes to sites of inflammation enabling the firm adhesion and diapedesis of leukocytes by interactions between integrins on leukocytes and adhesion molecules on the surface of endothelial cells. This mechanism has initially been thought to be solely dependent on the action of ICAM-1. Further studies have proven that the family of cell adhesion molecules (1, 2) comprises a wide range of proteins, which are subdivided into four major groups, i.e. selectins, mucin-like adhesion molecules (selectin ligands), integrins and members of the immunoglobulin superfamily. In acute and chronic inflammation the

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initial step lies in the recruitment of leukocytes. Thereby circulating leukocytes bind loosely to stimulated or injured endothelium via interactions between various selectins (L-, P-, E- selectin) and their ligands. Consequently, these leukocytes start to roll along the endothelium, a situation characterised by the repeated forming and breaking of loose bonds between adhesion molecules on the surface of each cell type. This process is accompanied by an increased secretion of cytokines by the endothelial cells leading to the activation of leukocytes, which in response strengthen their integrin adhesiveness (e.g. LFA-1, VLA-4). Subsequently, the rolling leukocytes become immobilised by the formation of firm interactions between leukocytes and the endothelium supported by ICAM-1, ICAM-2, VCAM-1 and MadCAM-1. Finally, the fixed leukocytes start to migrate across the endothelium while interacting with PECAM-1, ICAM-1 and VCAM-1 (3). Simultaneously, a wide variety of cytokines is released into circulation as a response of the cellular immune system to infections. The secretion of cytokines potentiates the immunological events and even induces the initiation of additional defense mechanisms, such as activation of the complement system and synthesis of acute-phase proteins in the liver (4). Since adhesion of leukocytes to vascular endothelium is the first step in their passage from blood to inflammatory sites, cytokines modulating the expression of adhesion molecules regulate leukocyte accumulation, and hence, the nature and progression of inflammatory responses (5, 6).

The *in vitro* model described herein deals with the treatment of HUVECs with two different cytokine combinations consisting of IL-2, IL-6, IFN-α, TNF-α plus either IL-8 or IL-4, IL-10 (final concentrations are listed in *Table I*). The first group of stimulants (IL-2, IL-6, IFN-α, TNF-α) is included in both cytokine mixtures tested in order to mimic the activation of endothelial cells by those cytokines present at elevated serum levels during rejection and infection processes (4). Combination I additionally contains IL-8, which is the major product secreted by activated monocytes and macrophages and has several effects on endothelial cells in culture that suggest that it may be an important angiogenic mediator stimulating the proliferation and migration of endothelial cells. Combination II also includes the anti-inflammatory cytokines IL-4 and IL-10, which are predominantely synthesised by Th2 cells. IL-4 demonstrates pivotal effects on endothelial cells by inducing VCAM-1 and inhibiting constitutive and increased ICAM-1 expression induced by IL-1, TNF-α or IFN-γ and E-selectin expression induced by IL-1 or TNF-α. IL-10 is known for its crucial role in the regulation of immune responses because it blocks activation of cytokine synthesis, and it is an inhibitor of macrophage, T cell and NK cell effector functions (7).

These cytokine mixtures are placed in the centre of our studies in order to elucidate their influence on the cell surface expression of a number of adhesion molecules on HUVECs (E-selectin, P-selectin, VCAM-1, ICAM-1, PECAM-1 and the L-selectin ligand CD 34), when combined in multi-component incubation cocktails, which is assessed by means of FACS.

Materials and Methods

Materials

Medium 199 (Hepes-modification) and heparin were ordered from Sigma-Aldrich, Steinheim, Germany. Penicillin/streptomycin, L-glutamine, trypsin/EDTA (0.05%/0.02% in Hanks balanced salt solution) and phosphate buffered saline (without Ca^{2+} and Mg^{2+}) were purchased from Gibco-Life Technologies, Merelbeke, Belgium. Cytokines used for stimulation were ordered from R&D Systems, Abington, UK. Fluorescence labelled (FITC, PE, PerCP) human monoclonal antibodies directed against PECAM-1, ICAM-1, E-selectin, P-selectin, VCAM-1 and the L-selectin ligand CD 34 were obtained from Serotec, Eching, Germany. Furthermore mouse IgG1 (Becton Dickinson, San Jose, CA, USA), ECGS (Upstate Biotechnologies, Waltham, MA, USA), human fibronectin (Chemicon, Hofheim, Germany), collagenase type 2 (Worthington Biochemical Corporation, Freehold, NJ, USA) and FCS (PromoCell, Heidelberg, Germany) were used.

Cell culture

Endothelial cells were isolated from human umbilical veins by use of a collagenase solution according to a standard procedure described elsewhere (8). Cells were seeded into T-75 culture flasks pre-coated with human fibronectin (9). The culture medium used consisted of Medium 199, FCS (20%), 10 000 U/mL penicillin, 10 000 μg/mL streptomycin, 100 mg/L low molecular weight heparin, 3 mmol/L L-glutamine and 30 mg/L ECGS. Endothelial cells were cultured in a humidified incubator set at 37.4 °C and 5% CO_2. Only confluent monolayers of single isolates at first passage were used for the experiments described below.

Stimulation of endothelial cells

For all stimulation experiments with endothelial cells an incubation time of 16 hours was chosen. During this period of time ECGS and heparin were eliminated from the incubation medium. Cell were incubated after addition of one of the two cytokine combinations consisting of those components listed in *Table I*. Control samples were treated in the same way omiting cytokine stimulation. At the end of incubation cells were detached enzymatically applying a standard trypsinisation protocol optimised elsewhere (10). The expression of cell surface molecules (VCAM-1, ICAM-1, PECAM-1, E-selectin, P-selectin and the L-selectin ligand CD 34) was examined by FACS. Each experiment was performed in quadruplicate for five times.

Table I Composition of both cytokine combinations applied for the stimulation of HUVECs. The respective final cytokine concentrations are indicated in brackets.

Combination I	Combination II
IL-2 (20 ng/mL)	
IL-6 (20 ng/mL)	
IFN-α (100 ng/mL)	
TNF-α (10 ng/mL)	
IL-8 (20 ng/mL)	
IL-4 (20 ng/mL)	
IL-10 (20 ng/mL)	
IL-8 (20 ng/mL)	IL-4 (20 ng/mL) IL-10 (20 ng/mL)

FACS analysis

The levels of adhesion molecule expression on the cell surface of unstimulated and cytokine treated endothelial cells were analysed via FACS. Therefore fluorescence-conjugated mouse monoclonal antibodies directed against E-selectin (FITC), P-selectin (FITC), VCAM-1 (FITC), ICAM-1 (PE), PECAM-1 (PE) and CD 34 (PE) were used. Samples were incubated for 30 minutes at 4 °C with an excess of the respective antibody. MFI of surface molecule expression was recorded on a FACScan analyser (Becton Dickinson). Data obtained were compaired with the levels of expression on unstimulated cells serving as controls.

Statistical Methods

The results obtained are given as absolute MFI values or as percentage changes in MFI versus respective control value (= 100%) and are expressed as mean ± confidence interval. Statistical significance was evaluated by ANOVA analysis.

Results

The levels of adhesion molecule expression on the surface of unstimulated HUVECs are summarised in *Table II*. These results are presented as MFI of surface molecule expression. The effects of incubating the cell monolayers with two different cytokine combinations are compared to the respective control niveau of unstimulated cells and are given as percentage changes in MFI ± confidence interval (%). Data are listed in *Table III*.

Under the incubation conditions applied (16 hours; 37.4 °C, 5% CO_2) E-selectin surface expression was statistically significant up-regulated when treated with one of the both cytokine combinations evaluated. Stimulating the cells with either mixture I or II resulted in a significant up-rise in E-selectin surface expression compared to control values giving percentage changes in MFI of 1029 ± 51% ($p<0.001$) and 864 ± 47% ($p<0.05$), respectively.

Table II MFI of surface molecule expression on unstimulated endothelial cells. Results are expressed as mean value ± confidence interval[a].

Cell surface molecule	Control
ICAM-1	566.6 ± 100.21
VCAM-1	20.96 ± 10.25
PECAM-1	710.18 ± 375.74
E-selectin	7.48 ± 2.88
P-selectin	5.21 ± 0.98
CD 34	20.46 ± 24.71

[a] ... confidence interval for n = 5; t (P, f) = 2.78; P = 0.95.

Table III Effects of different cytokine combinations on the expression of adhesion molecules on the cell surface of cultured HUVECs. Results are expressed as percentage changes in MFI versus respective control value (= 100%) and are expressed as mean ± confidence interval[a].

Cell surface molecule	Combination I	Combination I
ICAM-1	306 ± 28*	262 ± 26*
VCAM-1	573 ± 31*	734 ± 93*
PECAM-1	49 ± 4**	47 ± 2**
E-selectin	1029 ± 51**	864 ± 47**
P-selectin	315 ± 41*	260 ± 32*
CD 34	78 ± 10**	72 ± 8**

a ... confidence interval for n = 20; t (P, f) = 2.09; P = 0.95; significance compared to the controls:
*... $p<0.05$, **... $p<0.001$.

VCAM-1 surface expression was also significantly increased after treatment of cells with either cytokine combination I (573 ± 31%, $p<0.05$) or combination II (734 ± 93%, $p<0.05$). Furthermore, the application of these *in vitro* conditions also gave rise to an increased level of ICAM-1 expression resulting in 306 ± 28% for combination I and 262 ± 26% in the case of combination II ($p<0.05$, respectively).

Concomitantly, P-selectin expression was significantly up-regulated giving 315 ± 41% ($p<0.05$) after stimulation with combination I and 260 ± 32% ($p<0.05$) when cells were treated with combination II. In contrary to these observations PECAM-1 expression and CD 34 expression were both significantly down-regulated when recorded after treatment with either combination I or II. PECAM-1 expression was reduced to 49 ± 4% and 47 ± 2%, respectively ($p<0.001$), while CD 34 expression decreased to 78 ± 10% and 72 ± 8%, respectively ($p<0.001$).

Discussion

The present study dealt with the effects of cytokine stimulation on adhesion molecule expression on the cell surface of activated HUVECs. In our *in vitro* experiments we used two specific cytokine combinations combination I containing IL-2, IL-6, IL-8, IFN-α, TNF-α and combination II including IL-2, IL-4, IL-6, IL-10, IFN-α, TNF-α which were both found in sera of patients during inflammatory processes (4 6). Taking into consideration that organ rejection after transplantation is an inflammatory process, the effects of cytokines released during this process may be helpfull in the understanding of immunological mechanisms. *Figure 1* depicts a schematic illustration of the cytokine network during allograft rejection. In case of acute infections and rejections proliferating leukocytes migrate from the blood to the tissue, a process involving the interactions between granulocytes, lymphocytes, monocytes and endothelial cells. This process is mediated by the action of cell adhesion molecules. Since adhesion of leukocytes to vascular endothelium is the first step in their passage from the bloodstream to the site of rejection, cytokines influencing the expression of adhesion molecules may also regulate leukocyte accumulation, and hence, the nature and progression of inflammatory responses (11, 12).

We have demonstrated that stimulation with both combinations significantly up-regulates P-selectin expression, while single cytokine incubation failed to induce P-selectin expression as described in our previous work (13). A possible explanation for that could be that mixtures of cytokines may modulate a single receptor complex. Finally, expression of both E- and P-selectin is of particular importance since these molecules are responsible for the first contact between leukocytes and endothelial cells during inflammatory processes (14).

We have also found that incubating HUVECs with either combination I or combination II significantly up-regulates the expression of E-selectin. By comparing these data with results obtained for single TNF-α incubation (13), it can be pointed out, that both cytokine mixtures reduce the up-regulation of E-selectin surface expression (15, 16). Our previous work has already shown that no other cytokine in combination I has an influence on E-selectin expression when solely incubated with HUVECs (13). The attenuated effect on E-selectin expression under treat-

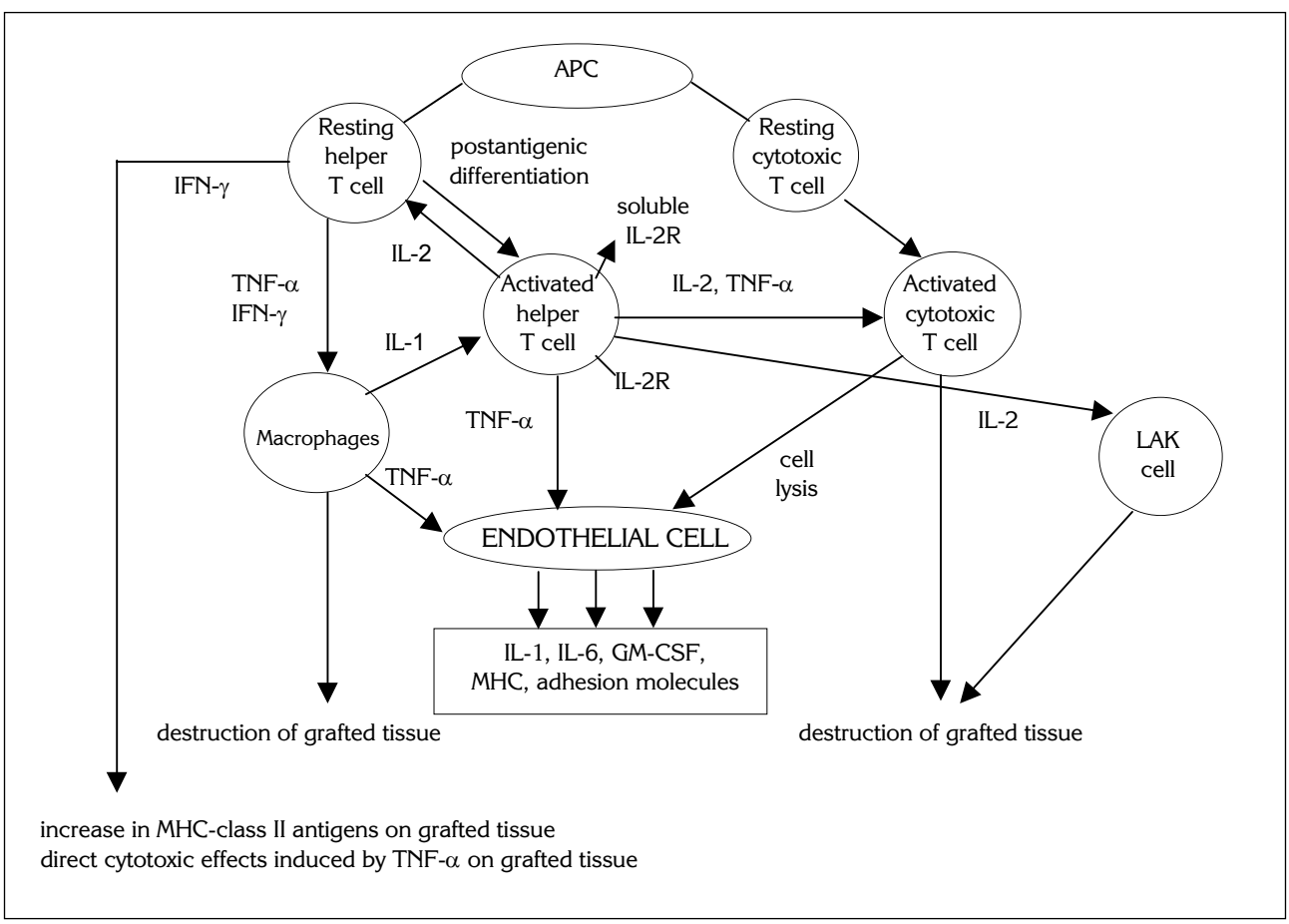

Figure 1. Schematic presentation of the cytokine network during allograft rejection.
Abbreviations: APC antigen presenting cell, LAK cell lymphokine activated killer cell.

ment with combination II might be attributed to the influence of the anti-inflammatory cytokines IL-4 and IL-10 within this mixture (17).

It has been illustrated in a number of reports that single cytokine incubation with either TNF-α, IFN-α, IL-1α or IL-4 up-regulates the expression of VCAM-1 (1, 13, 18). We have shown that incubating HUVECs with either combination I or combination II amplifies VCAM-1 expression on the cell surface, whereby combination II influences VCAM-1 expression to a slightly higher extent than combination I. These different outcomes of combination I and combination II could be explained by the fact that combination II is not only containing IFN-α and TNF-α but also IL-4, which is known to demonstrate crucial effects on endothelial cells not only by inducing VCAM-1 but also via the inhibition of constitutive ICAM-1 expression induced by IL-1, TNF-α or IFN-α and E-selectin expression induced by IL-1 or TNF-α (7, 17).

Furthermore, a number of cytokines have been proven to be up-regulating ICAM-1 expression, whereby TNF-α makes the greatest impact on this molecule (1, 18). We have examined the effects of combination I and II on the expression of this adhesion molecule and revealed that combination II has a minor effect on ICAM-1 expression probably because of the presence of IL-4 in the incubation cocktail.

In conclusion, this *in vitro* model dealt with the effects of two specific cytokine combinations on the expression of surface molecules on HUVECs. It has been demonstrated that both combinations led to a significant increase in VCAM-1 expression, to a slight amplification of ICAM-1 and to the induction of E- and P-selectin. Our data emphasize the importance of endothelial cells during inflammatory processes expressing adhesion molecules, which are indispensable for leukocyte transmigration.

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EFEKTI KOMBINACIJE CITOKINA NA ISPOLJAVANJE ADHEZIVNIH MOLEKULA NA ENDOTELSKE ĆELIJE HUMANE UMBILIKALNE VENE U *IN VITRO* MODELU INFLAMACIJE

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Kratak sadržaj: Kao esencijalni rani događaj pri aktivaciji imunskog sistema nađeno je povećanje pripajanja cirkulišućih neutrofila, limfocita i monocita na mikrovaskularni endotel. Ovo je praćeno migracijom pomenutih ćelija kroz zidove krvnih sudova i njihovom akumulacijom na mestima oštećenja tkiva. Ovaj proces je omogućen specifičnim ćelijskim adhezivnim molekulama, koje su ključne za imunski i inflamatorni odgovor. U ovom radu ispitan je uticaj stimulacije citokinana na endotelske adhezivne molecule nakon stimulacije endotelskih ćelija humane umbilikalne vene (HUVECs) sa dve specifične kombinacije citokina. Obe kombinacije sadržale su IL-2, IL-6, IFN-α and TNF-α, koji su povišeni u krvi pacijenata tokom reakcije odbacivanja organa nakon transplantacije ili infekcije. Kombinacija i dodatno je sadržala IL-8 koji oslobađaju aktivirani monociti i makrofage i koji se smatra medijatorom angiogeneze stimulišući proliferaciju i migraciju endotelskih ćelija. S druge strane, kombinacija II sadržala je dva anti-inflamatorna citokina IL-4 i IL-10 koji se uglavnom stvaraju u Th2 ćelijama. Dok IL-4 ispoljava višestruke stimulatorne i regulatorne efekte, IL-10 ima ključnu ulogu u regulaciji imunskog odgovora. Oba citokina blokiraju sintezu citokina kao što su IL-1, TNF-α i IL-12 koji u početku inflamatornih procesa imaju važnu regulatornu ulogu. Ove mešavine citokina imaju centralno mesto u radu pri određivanju uticaja na ispoljavanje brojnih adhezivnih molekula na HUVECs tokom multi-komponentne stimulacije. Primenom pomenutih kombinacija citokina dobijeni komparativni efekti statistički značajnog povećanja srednjeg intenziteta fluorescencije (MFI) VCAM-1, slabijeg povećanja ICAM-1 molekula praćeno indukcijom E- i P-selektina. Ove molekule imaju ključnu ulogu u procesu transmigracije leukocita. Eksperimenti su pokazali veliki efekat povećanja antigena na ćelijskoj površini u uslovima u kojima se simulira inflamacija. Ovo ukazuje na značaj endotelskih ćelija tokom aktivacije imunskog sistema.

Ključne reči: endotelske ćelije; adhezivne molekule; citokini; inflamacija.

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