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Function and Regulation of the Vascular Cell Adhesion Molecule-1 in TNF-Stimulated Endothelial Cells

Funktie en regulatie van het vasculaire cel adhesie molecuul-1 in endotheelcellen gestimuleerd door TNF

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CONTENTS

1 General Introduction

The accumulation of circulating monocytes in the vessel wall is an important aspect of the pathogenesis of atherosclerosis and allied problems such as restenosis after percutaneous transluminal angioplasty (PTA). Three distinct steps can be distinguished in the stimulated accumulation of these cells. First there is an increased rolling, followed by firm adhesion, and finally migration of the monocytes in the direction of a chemotactic gradient. These different steps are regulated by specific endothelial cell adhesion molecules. In general rolling is mediated by the selectins, while immunoglobulin supergene family member vascular cell adhesion molecule (VCAM)-1 specifically mediates rolling and firm adhesion of monocytes. VCAM-1 may thus enhance the local accumulation of monocytes even in the absence of selectin expression. In experimental models of atherosclerosis VCAM-1 expression is an early feature of the atheromatous lesion, which suggests that VCAM-1 plays a role in the initiation and progression of the atherosclerotic plaque. The expression of VCAM-1 on endothelial cells is induced by the cytokine tumor necrosis factor α (TNF) and presumably regulated at the transcriptional level in part through the activation of the transcription factor nuclear factor (NF) κB. TNF stimulates intracellular signalling in endothelial cells through the production of lipid second messengers and mitogen activated protein kinase cascades. It has been hypothesized that reactive oxygen species (ROS), such as hydrogen peroxide, function as intracellular second messengers in the signal transduction of TNF. This putative role for ROS may comprise a novel mechanism of intracellular signalling next to protein phosphorylation, the cyclic nucleotide system, and signalling through the inositol phosphates and intracellular calcium. In the present thesis we have investigated the possible role of ROS as second messengers in the TNF stimulated expression of VCAM-1 by endothelial cells. (*)

* References see Chapter 5

2 Atherosclerosis as a response of the vascular wall to tissue injury

Atherosclerosis and its complications, such as myocardial infarction, stroke, and also allied problems like restenosis remain a major cause of morbidity and mortality in Western societies. For example in the Netherlands ischemic heart disease alone accounted for 15% of the total mortality of men and woman in 1995 (1).

Atherosclerosis is a slowly progressive disease of the elastic and medium-sized muscular arteries, which generally begins in childhood and does not become manifest until middle-age or later. Its pathogenesis still has not been completely elucidated (2). The complexity of the atherosclerotic lesion, i.e., the atheromatous or fibro-fatty plaque has certainly attributed to this fact. Although plaques exhibit histologic variability a cellular fibro-fatty plaque in general consists of 1) a fibrous cap with mostly smooth muscle cells, some leukocytes and a dense connective tissue, 2) a cellular area including macrophages, smooth muscle cells, and T-lymphocytes, 3) a deeper necrotic core with cellular debris, extracellular lipid droplets, cholesterol crystals, and calcium deposits. This area contains the numerous lipid-laden cells, also called foam cells, of the macrophage and smooth muscle cell origin (2,3). Finally, in the periphery of the lesion sometimes neovascularization can be observed (2). On the basis of this histologic evidence it has been argued that plaques and sites of inflammation have a number of features in common. These include leukocyte infiltration, mesenchymal cell proliferation and fibrosis.

In 1976 Ross and Glomsett formulated their theory of atherosclerosis as a response of the vascular wall to tissue injury (4). This theory was based in part on the observation that experimentally induced local denudation of the endothelium ultimately gives rise to lesions that closely resemble the atheromatous plaque (4). However, endothelial denudation is not a consistent early feature of one important form of atherosclerosis, the one initiated by hypercholesterolemia alone (5). In the absence of endothelial denudation more subtle changes in the endothelium, such as injury produced by toxins and viruses, may also prompt the response to tissue injury (6). This type of injury is referred to as non-denuding injury. The endothelial cell responds to non-denuding injury by changes in function or by the induction of new endothelial properties, such as the increased endothelial interaction with circulating monocytes (7,8). The adherence of circulating monocytes (and lymphocytes) to the arterial endothelial lining is one of the earliest detectable events in experimental atherosclerosis (2,6). The subsequent transendothelial migration and accumulation in the intima, the transformation

of monocytes into foam cells and the secretion of cytokines and growth factors by these cells are important events in the initiation and progression of the atherosclerotic plaque (6,9) . Therefore in order to interfere with these processes it is of importance to establish the mechanism whereby monocytes accumulate into the tissue.(*)

* References see Chapter 5

3 Interaction of endothelial cells with circulating leukocytes

Under non-pathological conditions there is a limited interaction of marginating monocytes with endothelial cells. Monocytes and also granulocytes roll along the endothelial lining due to the shear force of the circulation and the making and breaking of ionic bonds. The interaction of these leukocytes is dramatically increased under inflammatory conditions through the increased expression of specific adhesion molecules. These adhesion molecules are selectins, immunoglobulin supergene family members, and integrins (Table 1).

Selectins are single chain glycoproteins with an N-terminal lectin domain, an epidermal growth factor (EGF)-like domain, a varying number of short consensus repeat motifs (structurally related to proteins that regulate complement activation), a transmembrane domain, and a short cytoplasmic tail (fig 1, page 8). The selectins include L-selectin expressed on monocytes and granulocytes (10), and P- and E-selectin expressed by endothelial cells. All three selectins appear to recognize sialylated carbohydrate structures on their counterreceptors, which are related to the sialylated Lewis^a and Lewis^x tetrasaccharides (11). For L- and P-selectin these ligands have been identified as mucin-like molecules, which are serine- and threonine-rich proteins that are heavily O-glycosylated. L-selectin recognizes two mucins, i.e., the glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) and CD34, the latter being expressed on the surface of vascular endothelial cells (11,12). P-selectin recognizes the P-selectin glycoprotein ligand (PSGL-1) or CD162 expressed by monocytes and granulocytes (13,14). Like P-selectin E-selectin can interact with both monocytes and granulocytes (15). P-selectin is stored in the Weibel-Palade bodies of the endothelial cells and is rapidly (within 5 - 20 min) translocated to the endothelial membrane after stimulation with histamine or thrombin (10). And E-selectin is induced in endothelial cells after 2 to 4 h of stimulation with the inflammatory cytokines TNF and interleukin-1 (IL-1) (10). Exposure to lipopolysaccharide (LPS) will also stimulate the expression of E-selectin.

Members of the immunoglobulin supergene family have a variable number of immunoglobulin-like domains. The intercellular cell adhesion molecule (ICAM)-1, ICAM-2 and the vascular cell adhesion molecule (VCAM)-1, respectively have two, five and six (or seven) of these domains (17). ICAM-1 and ICAM-2 both are expressed by resting endothelial cells. The expression of ICAM-1, but not ICAM-2, is enhanced after stimulating endothelial cells for 4 to 6 h with the cytokines TNF and IL-1 (17,18). VCAM-1 is absent on the surface

Table 1: Adhesion molecules involved in leukocyte- endothelial cell interactions.

Endothelial receptor	Function	Basal expression	Inflammatory mediators	Maximal expression after activation	Ligands on leukocyte
P-selectin	Rolling	no	histamine, thrombin	5-30 min	PSGL-1
E-selectin	Rolling	no	IL-1, TNFα, LPS	4-6 h	sialyl Lewis$^{a/x}$ like structures
VCAM-1	Rolling/Firm Adhesion/Extravasation	no	IL-1, TNFα, IL-4, LPS	12-24 h	α4β1- and α4β7 integrins
ICAM-1	Firm adhesion/ Extravasation	yes	IL-1, TNFα, LPS, IFNγ	12-48 h	LFA-1, CR3
ICAM-2	Firm adhesion	yes	none*		LFA

*The expression of ICAM-2 on human umbilical vein endothelial cells is refractory to stimulation by the inflammatory cytokines TNF, and IL-1.

of resting endothelial cells and transcriptionally induced after 6 hours of stimulation with TNF and IL-1 (19). In contrast to E-selectin the stimulated expression of both VCAM-1 and ICAM-1 is sustained, and remains elevated for more than 24 hours (Table I) (20). VCAM-1, ICAM-1 and also ICAM-2 binds to integrin molecules expressed by monocytes and granulocytes.

Integrin adhesion molecules consist of an α and β-chain that are covalently linked. Subfamilies can be distinguished on the basis of their β-chain. The β2 integrins are expressed exclusively by leukocytes and essential for firm adhesion of monocytes and granulocytes to endothelial cells. They include the αLβ2 (CD11a/CD18) or leukocyte function associated antigen-1 (LFA-1), αMβ2 (CD11b/CD18) or complement receptor type-3 (CR3), and αXβ2 (CD11c/CD18) (15,21). The ligands for CD11a/CD18 are the intercellular cell adhesion molecule (ICAM)-1 (CD54) and ICAM-2 (CD102). CD11b/CD18 integrin also binds ICAM-1. CD11b/CD18 is stored in peroxidase-negative granules of granulocytes and monocytes and can be rapidly translocated to the surface of these cells after stimulation, for example with N-formyl peptides (22). These peroxidase-negative granules also contain CD11c/CD18 (22). CD11c/CD18 is markedly expressed on monocytes and only moderately on granulocytes. Its ligand on endothelial cells has not yet been identified. The β1 integrin α4β1 (VLA-4) is specifically involved in the accumulation of chronic inflammatory cells. VLA-4 is expressed by monocytes and specifically mediates the firm adhesion of chronic inflammatory cells (monocytes and lymphocytes) to VCAM-1 (23). VCAM-1 can also recruit lymphocytes through binding the α4β7 integrin (17).

The stimulated accumulation of circulating leukocytes has been characterized as a three-step phenomenon (17). Initially the circulating leukocyte is tethered to the vessel wall and engaged in rolling adhesions mediated by the selectins. Tethering brings the leukocytes into close proximity with chemoattractants that are being produced in the vessel wall under inflammatory conditions and will enhance the exposure of the leukocyte to chemoattractants. These chemoattractants bind to G-protein coupled receptors on the leukocyte surface, which transduce signals that activate the integrin (24). Due to a conformational change in the integrin the adhesiveness for its counterreceptor, a member of the immunoglobulin supergene family, will be increased and firm adhesion of the leukocyte will be established (17,25). Ultimately the leukocyte will cross the endothelial lining of the blood vessel, directed by a chemotactic gradient, and enter the tissues through interactions of the integrins with members of the immunoglobulin supergene family (26). This last step is called extravasation.

Untill recently it was thought that the first step or tethering and rolling was entirely mediated by the selectins. However, in addition to mediating firm adhesion a recent observation suggests that VLA-4 can also mediate tethering and rolling on VCAM-1 (27). Thus prior to activation and tight adhesion, VLA-4 could support tethering and rolling of monocytes on VCAM-1. Therefore in the absence of P- and E-selectin monocytes but not (neutrophilic) granulocytes can still be accumulated at the site of tissue injury. The selective accumulation of monocytes is particularly relevant for the development and progression of atherosclerotic disease. In experimental models of atherosclerosis VCAM-1 expression is an early feature of the lesion (28,29). Therefore in order to interfere with the pathophysiology of atherosclerosis it is of importance to define the intracellular signalling mechanism of VCAM-1 expression. (*)

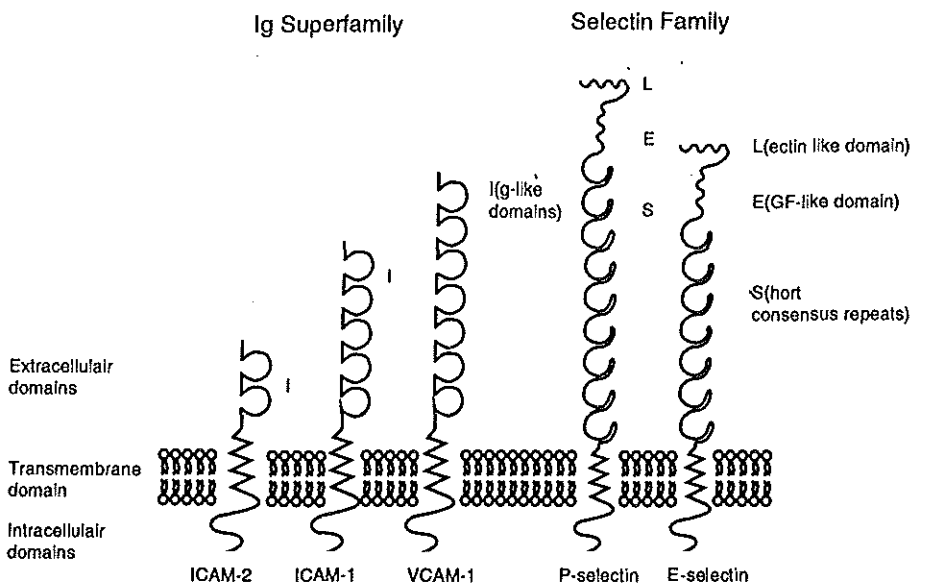

Fig 1. The basic molecular structure of the members of the Ig superfamily and the selectin family of leukocyte adhesion molecules expressed by the vascular endothelium.

* References see Chapter 5

4 The regulation of VCAM-1 expression on the vascular endothelial lining

VCAM-1 is upregulated by the inflammatory cytokines TNF and IL-1 which stimulate intracellular signalling through distinct receptors. On endothelial cells IL-1 interacts with the type I IL-1 receptor, and TNF with the p55 and p75 TNF receptors. The present thesis is focussed on the intracellular signalling mechanism involved in the expression of VCAM-1 on endothelial cells stimulated with TNF

4.1 Signal transduction from the TNF receptor

TNF elicits an array of responses like proliferation, differentiation, and apoptosis (programmed cell death) in many different target cells. In vivo TNF is a mediator of the inflammatory and immune responses. The wide range of activities induced by TNF is explained by the presence of receptors for TNF (TNFR's) on almost all nucleated cell types. Two different receptors with overlapping activities have been identified, i.e. the 55 kD TNFR1 and the 75 kD TNFR2. Endothelial cells express both the TNFR1 and the TNFR2, but in most cases gene induction can be attributed to stimulation of the TNFR1 (30). The present description will concentrate on the responses elicited by the TNFR1.

Upon stimulation by TNF receptor clusters are formed, which are rapidly internalized and degraded. (31). The receptor is not recycled and therefore protein synthesis is required to maintain cell surface receptor number. Important progress has been made in understanding the mechanism whereby TNF activates signal transduction from the TNFR1. A number of intracellular proteins have been identified that associate with the clustered receptors. The recruited proteins belong to the family of TNF receptor-associated factors (TRAF's) and the death domain containing homologues (32). A schematic representation of these proteins and the cellular effects they elicit is depicted in Fig 1. The factors represented in this scheme have been identified in most cases by the yeast two hybrid screening method, a powerful technique to study protein-protein interactions (33). These proteins include TRADD, TRAF-2, and RIP. The fas associated protein with death domain (FADD) is recruited to the TNFR1, and has been linked specifically to the apoptotic response induced by TNF (34,35). The apoptotic response is beyond the scope of the present thesis, and therefore FADD will not be further discussed here. The other factors have been linked to the activation of the inducible transcription factor nuclear factor (NF) κB and activating protein-1 (AP-1) and will be introduced here briefly.

The TNF receptor-associated-death-domain (TRADD) binds the cytoplasmic domain of the TNFR1 via death domain interactions (36). Overexpression of TRADD results in the activation of NFκB (36). TRADD holds a central position in the intracellular signalling from the TNFR1 by serving as a platform for the recruitment of several other proteins (37). These include the receptor interacting protein (RIP) (38,39), and the TNF receptor associated factor (TRAF)-2 (34,40).

The TRADD interacting protein RIP has three distinct domains. A C-terminal death domain which interacts with TRADD, an intermediary region which is capable of activating NFκB, and a N-terminal kinase domain that may be dispensable for TNF-mediated NFκB activation (32,39). In addition to stimulating apoptosis and NFκB activation RIP is also capable of activating the MAP kinase JNK (37).

The TRAF superfamily member TRAF2 has a characteristic C-terminal TRAF domain. With this domain TRAF2 binds to TRADD. TRAF-2 also binds to the TNFR2, which may explain some of the overlapping effects of the two TNF receptors (37). TNF-induced NFκB activation requires TRAF-2 (34). And transient expression of TRAF-2 results in the activation of the MAP kinase JNK, but does not mediate apoptosis (37). Like TRADD TRAF-2 is thought to function as an adaptor protein that recruits other proteins to the TNFR1 complex. Three TRAF-2 interacting proteins have been identified, which are all involved in the activation of NFκB. These proteins and their role in the activation and inhibition of NFκB will be discussed in the next paragraph.

4.2 Activation and inhibition of the transcription factor NFκB

NFκB is a pleiotropic regulator of genes involved in immune and inflammatory responses. NFκB refers to a family of transcription factors that form homo and heterodimeric complexes with distinct transcriptional activity (41,42). The DNA binding activity of NFκB is regulated by the inhibitory IκB protein family. In the absence of a suitable stimulus NFκB is retained in the cytosol, complexed to IκB. Following activation by TNF IκB is phosphorylated which targets the complex for degradation in the proteasome proteolytic pathway (43). After proteolytic degradation of IκB, NFκB translocates to the nucleus to activate gene transcription.

Recently the kinases involved in the phosphorylation of IκB have been identified. These include the MAP kinase kinase kinase (MAP3K) NFκB inducing kinase (NIK) and the IκB

kinase . The identification of NIK was an important link in establishing the NFκB activation pathways downstream of the TRAF family (44). NIK associates with TRAF2, and is dedicated to NFκ B activation but does not phosphorylate IκB itself. The putative IκB kinase was identified recently by Regnier et al (45). This kinase displays the characteristics expected of a cytokine-inducible IκB kinase. It associates with the IκB complex and specifically phosphorylates IκB on both serine 32 and serine 36, these modifications are required for the signal-induced degradation of IκB (45).

In addition to stimulatory effectors TRAF2 may also recruit inhibitors of NFκB activation to the TNF receptor. Overexpression of the TRAF2 interacting protein A20 for example inhibits the activation of NFκB (46). The transcriptional activation of A20 itself depends upon κB elements (47). This may be a mechanism of feedback inhibition in the activation NFκB. The interacting TRAF (I-TRAF) also inhibits the activation of NFκB stimulated by TNF. However, the role of I-TRAF may be more complex, since at suboptimal concentrations I-TRAF potentiates the activation by NFκB induced by TRAF2 (32).

The protein recruitment model discussed here has generated important insight into how the signalling processes emanating from the TNFR1 may lead to the activation of NFκB. However, a physiological role for these proteins remains to be demonstrated. Also, the proteins that associate with the TNF receptor have mostly been identified in other cells than the endothelial cell. Although it is not unlikely that the model discussed here applies to the endothelial cell as well, it has been stressed that TNF-induced signalling mechanisms may be cell type dependent (31). Therefore it is required to focus on the signalling events that have been demonstrated in the endothelial cell as well.

4.3 Downstream effectors involved in the endothelial VCAM-1 expression

In endothelial cells a number of downstream effectors involved in the expression of VCAM-1 have been identified. These include lipases, kinases, and lipid second messengers.

The phosphatidylcholine specific phospholipase C (PC-PLC) may be one of the first enzymes activated by the TNF receptor. PC-PLC hydrolyses PC and generates the lipid second messenger diacylglycerol (DAG). DAG activates an acid sphingomyelinase (aSM-ase) leading to the formation of ceramide, a lipid second messenger that activates NFκB in Jurkat cells (48). In endothelial cells the TNF stimulated VCAM-1 expression can be inhibited by the specific PC-PLC inhibitor D609. This may suggest that PC-PLC and ceramide are

involved in the expression of VCAM-1 (49). However, although the production of small amounts of ceramide in TNF stimulated endothelial cells has been confirmed, DAG accumulation could not be demonstrated in these cells (50). Therefore no definitive conclusion can be drawn on the role of PC-PLC in the endothelial response to TNF.

Ceramide can also be produced by a second sphingomyelinase with a neutral pH optimum (nSM-ase). The nSM-ase is activated through its interaction with FAN or 'factor associated with nSM-ase activation', which directly couples the nSM-ase to the TNFR1 (51) (see Fig 1, page 14). The nSM-ase is localized in the cell membrane. In contrast to the aSM-ase, which is localized in the endosomal compartment, nSM-ase is not involved in the activation of NFκB (52). Apparently the action of ceramide is determined by the subcellular site of its production (52). It has been proposed that the nSM-ase activity is linked to the activation of MAPK cascades (53,54).

MAPK cascades exist of a MAP kinase kinase kinase or MAP3K, a MAP2K, and a MAPK, which is the most downstream effector of the cascade. MAPK's are activated by dual phosphorylation on tyrosine and threonine residues, and can be grouped into two different classes, which are the extracellularly regulated kinases (ERK's) and the stress kinases JNK and p38 (55-59). In general the ERK's are activated by growth factors, whereas the stress kinases respond to pro-inflammatory cytokines and environmental stresses (59). However, in endothelial cells TNF rapidly activates ERK 1, ERK 2, p38 and JNK (50).

The MAP3K Raf-1, ERK1 and 2, but not p38 and JNK, are rapidly activated in endothelial cells treated with SM-ase. Therefore it has been postulated that the ERK pathway in endothelial cells is ceramide dependent and the p38 and JNK pathway are not (50). In the presence of SM-ase the expression of E-selectin and VCAM-1 respectively amounted to 40 %, and 5% of the control stimulated with TNF (50). Therefore activation of the ceramide dependent pathway seems to be of greater importance for the expression of E-selectin than of VCAM-1.

Stimulation of the MAPK ERK leads to the phosphorylation and activation of the CCAAT enhancer binding protein C/EBP, while p38 and JNK have been linked to the activation of the 'activating transcription factor' (ATF) family of transcription factors ATF-2 and c-jun (60). The contribution of the ATF and NFκB family members to the regulation of VCAM-1 gene expression will be discussed next.

4.4 The transcriptional activation of VCAM-1 gene

The VCAM-1 gene gives rise to two alternatively spliced forms, that differ in their number of integrin binding sites (15). On the surface of human umbilical vein endothelial cells only the 110 kD polypeptide can be detected, which is consistent with the seven (immunoglobulinlike) domain form of VCAM-1 (61). To determine whether the increase in VCAM-1 expression in response to TNF is mediated by transcriptional initiation of the gene nuclear run-off experiments have been performed (62). VCAM-1 transcriptional activity is not detectable in uninduced human umbilical vein endothelial cells but is markedly increased as early as 2 h after stimulation with TNF. Indicating that the TNF-stimulated expression of VCAM-1 is regulated mainly at the transcriptional level (62).

To study the mechanism of the transcriptional activation of VCAM-1 expression the structural organization of the VCAM-1 promotor has been characterized. The promotor contains cytokine-dependent enhancers as well as negative regulatory elements or silencers (62,63). These silencers are localized in the –2167 bp to –755 bp region of the 5' flanking sequence of the VCAM-1 gene (62). TNF stimulation of endothelial cells can overcome the effect of the(se) silencer(s) and activate VCAM-1 expression (62). Whithin the –755 bp to –2 bp 5' flanking region consensus sequences have been identified for at least four families of transcription factors. These include seqences that conform to consensus GATA-elements (62,64), a tandem binding site for NFκB (62,63), an interferon regulatory factor (IRF)-1 binding element (65), and an activator protein (AP)-1 consensus sequence (62). Except for the AP-1 site all these sequences are functional (62-66). Proteins binding to these elements may interact to stimulate transcription of the VCAM-1 gene. Structural alteration of the AP-1 site does not diminish basal or TNF-stimulated VCAM-1 reporter gene expression in bovine aortic endothelial cells (62). Therefore binding of the c-fos-c-jun complex to the AP-1 site does not seem to be required for efficient VCAM-1 gene expression in endothelial cells.

An intriguing aspect of VCAM-1 gene expression is the fact that it is specifically regulated by a redox sensitive step. In contrast to ICAM-1, VCAM-1 be can inhibited by the antioxidants pyrrolidine dithiocarbamate (PDTC) and N-acetylcysteine (NAC) at the transcriptional level (67). This may suggest that the regulation of VCAM-1 gene expression is coupled to oxidative stress through reduction-oxidation (redox) sensitive transcriptional regulatory factors (67). Oxidative stress, or the deleterious effects of reactive oxygen species generated in aerobic metabolism, is counteracted through cellular anti-oxidants and anti-oxidant

enzymes, for example glutathione (GSH) and GSH peroxidase. A change in the balance of the production and interception of the reactive oxygen species (ROS) may have an intracellular signalling function, which would imply that ROS function as intracellular second messengers. (*)

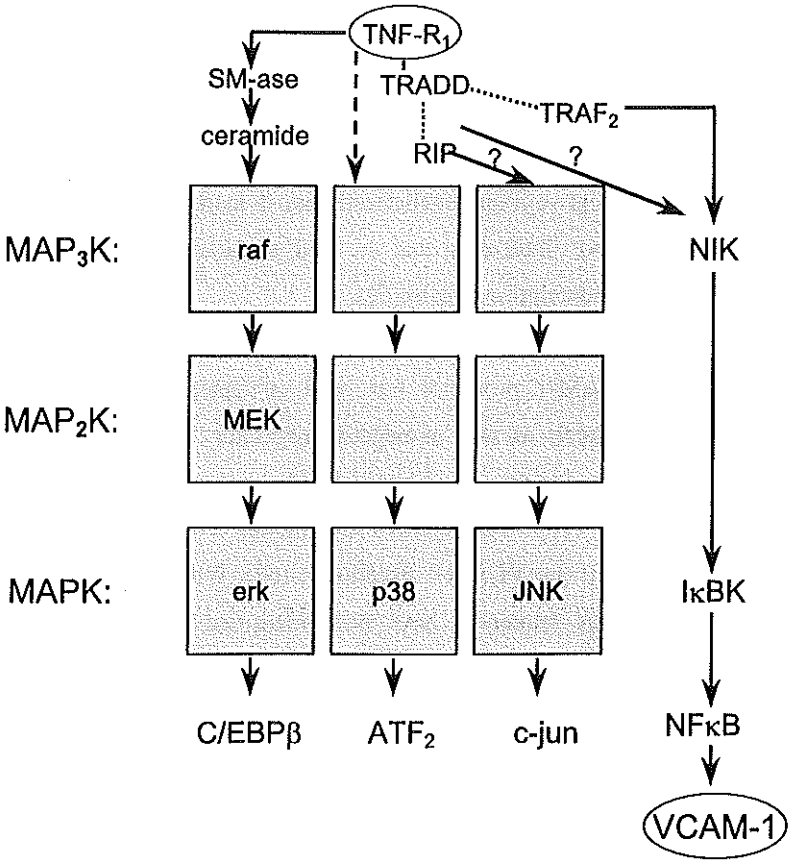

Fig 1. TNF stimulates the mitogen activated protein kinases (MAPK) extracellulary regulated kinase (erk), p38, and the c-jun kinase JNK in Endothelial Cells. These downstream effectors of the different MAP kinase cascades activate the transcription factors CCAAT enhancer binding protein C/EBPβ, activating transcription factor (ATF)-2 and c-jun. Activation of the transcription factor nuclear factor (NF) κB through the NFκB inducing kinase (NIK) and the inhibitory IκB kinase (IκBK) is requisite for the transcriptional induction of VCAM-1.

* References see Chapter 5

5 Aim of the thesis

ROS (superoxide anion, hydrogen peroxide and hydroxyl radical) are well known extracellular mediators of inflammation. Phagocytes, involved in host defence, generate ROS by the inducible phagocytic NADPH-oxidase in a strictly controlled manner. The superoxide anion has long been considered as a destructive entity only. However, cells not involved in host defence, such as smooth muscle cells secrete superoxide and may influence biological effects like vascular relaxation (68). Based on this and other findings it was hypothesized by Saran and Bors that the superoxide anion is a specific biological messenger as well (69). In fact in bacterial cells gene expression can be modified by an increased production of ROS (70,71). The superoxide anion is thought to induce a redox-induced conformational change of the transcription factor soxR, which will activate transcription from the soxS gene. The soxS protein, which is a transcription factor also, then stimulates transcription of a number of defence proteins (72). In addition to the ROS superoxide anion hydrogen peroxide has been shown to stimulate gene expression as well. Hydrogen peroxide activates the transcription factor oxyR through direct oxidation, resulting in the expression of genes involved in the protection of the bacterial cell from oxidative damage (73) Could similar ROS driven mechanisms be operative in eukaryotic cells? A number of observations indeed point into that direction.

In the Jurkatt T lymphoma cell line activation NFκB was activated by the additon of hydrogen peroxide in the mM range. Furthermore, various antioxidants and metal chelators, e.g., PDTC, NAC, and desferrioxamine, inhibited the activation of NFκB (74,75). However, since both the VCAM-1 and ICAM-1 promotors contain consensus NFκB binding sites, these findings do not explain the specific sensitivity of VCAM-1 expression to redox regulation. Therefore other redox sensitive signal transduction pathways must be involved in the expression of VCAM-1. In this respect, it is noteworthy that hydrogen peroxide results in a 10 -14 fold activation of the p38 MAPK pathway in endothelial cells (76). Whether this could be relevant for the expression of VCAM-1 has not been determined as yet.

In the present thesis we have investigated the role of ROS and the p38 MAPK in the TNF-stimulated endothelial expression of VCAM-1 (chapter six to eight). In addition we have investigated in chapter nine if activated monocytes play a significant role in the patho-physiology of restenosis after PTA in humans.

References

1. Dalstra JAA, Reitsma JB. Hart-en vaatziekten in Nederland. Cijfers over ziekte en sterfte. P7-p16 Den Haag 1997.
2. Munro JM, Cotran RS. The pathogenesis of atherosclerosis and inflammation. Laboratory Investigation. 1988;58:249-261
3. Aqel NM, Ball RY, Waldman H, Mitchinson MJ. Identification of macrophages and smooth muscle cells in human atherosclerosis using monoclonal antibodies. J Pathol 1985;146:197-204
4. Ross R, Glomsett JA. The pathogenesis of atherosclerosis. N Engl J Med. 1976;295:369-425
5. Davies PF, Reidy MA, Goode TB, Bowyer. Scanning electron microscopy in the evaluation of endothelial integrity of the fatty lesions in atherosclerosis. Atherosclerosis. 1976;25:125
6. Ross R. The pathogenesis of atherosclerosis, an update. N Eng J Med. 1986;314:488-500
7. Gerrity RG. The role of the monocyte in atherogenesis. I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am J Pathol. 1981;103:181-190
8. Faggiotto A, Ross R, Harker L. Studies of hypercholesterolemia in the nonhuman primate. I. Changes that lead to fatty streak formation. Arteriosclerosis. 1984;4:323-340
9. Cybulsky MI, Gimbrone MA. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science. 1991;251:788-791
10. Welply JK, Keene JL, Schmuke JJ, Howard SC. Selectins as potential targets of therapeutic intervention in inflammatory disease. Biochim Biophys Acta. 1994;1197:215-226
11. Lasky LA. Selectins: interpreters of cell-specific carbohydrate information during inflammation. Science. 1991;258:964-969
12. Baumhueter S, Singer MS, Henzel W, Hemmerich S, Renz M, Rosen SD, Lasky LA. Binding of L-selectin to the vascular sialomucin, CD34. Science. 1993;262:436-438
13. Moore KL, Stults NL, Diaz S, Smith DF, Cummings RD, Varki A, McEver RP. Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. J Cell Biol.1992;118:445-456
14. Sako D, Chang XJ, Barone KM, Vachino G, White HM, Shaw G, Veldman GM, Bean KM, Ahern TJ, Furie B, Cumming DA, Larsen GR. Expression cloning of a functional glycoprotein ligand for P-selectin. Cell. 1993;75:1179-1186
15. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell. 1994;76:301-314
16. McEver RP, Beckstead JH, Moore KL, Marshall-Carison L, Bainton DF. GMP-140 a platelet α-granule membrane protein, is also synthesized by vascular endothelial cell and is loclized in Weibel Palade bodies. J Clin Invest 1989;84:92-99
17. Springer TA. Adhesion receptors of the immune system. Nature. 1990;346:425-433
18. Wardlaw A. Leukocyte adhesion to endothelium. Clinical and Experimental Allergy. 1990;20:619-626
19. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NFκB and cytokine-inducible enhancers. FASEB J. 1995;9:899-909

20. Carlos TM, Schwartz BR, Kovach NL, Yee E, Rosso M, Osborn L, Chi-Rossos G, Newman B, Lobb R, Harlan JM. Vascular Cell Adhesion Molecule-1 mediates lymphocyte adherence to cytokine-activated human endothelial cells. Blood 1990, 76:965-970
21. Albelda SM. Endothelial and epithelial cell adhesion molecules. Am J Respir Cell Mol Biol. 1991;4:195-2031253
22. Miller LJ, Bainton DF, Borregaard N, Springer TA. Stimulated mobilization of monocyte Mac-1 and p150,95 adhesion proteins from an intracellualr vesicular compartment to the cell surface. J Clin Invest. 1987;80:535-544
23. Elices MJ, Osborn L, Takada Y,Crouse C, Luhowskyj, Hemler ME, Lobb RR. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell. 1990;60:577-584
24. Wu D, LaRosa GJ, Simon M. G protein-coupléd signal transduction pathways for inteleukin-8. Science. 1993;261:101-103
25. Landis RC, Bennett RI, Hogg N. A novel LFA-1 activation epitope maps to the I domain. J Cell Biol. 1993;120:1519-1527
26. Arfors KE, Lundberg C, Lindborm L, Lundberg K, Beatty PG, Harlan JM. A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo. Blood. 1987;69:338-340
27. Alon R, Kassner PD, Carr MW, Finger EB, Hemler ME, Springer TA. The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. J Cell Biol. 1995;128:1243-1253
28. Cybulski MI, Gimbrone MA. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science 1991;251:788-791
29. Li H, Cybulski MI, Gimbrone MA, Libby P. Inducible expression of vascular cell adhesion molecule-1 by vascular smooth muscle cells in vitro and within rabbit atheroma. Am J Pathol 1993;143:1551-1559.
30. Vandenabeele P, Declerq W, Beyaert R, Fiers W. Two tumour necrosis factor receptors: structure and function. Trends in Cell Biol. 1995;5:392-399
31. Beyaert R, Fiers W. Molecular mechanisms of tumor necrosis factor-induced cytotoxicity. What we do and do not understand. FEBS Letters 1994;340:9-16
32. Darnay BG, Aggarwal BB. Early events in TNFsignaling: a story of associations and dissociations. J Leukoc Biol. 1997;61:559-566
33. Fields S, Song O. A novel genetic system to detect protein-protein interactions. Nature. 1989;340:245-246
34. Hsu H, Shu HB, Pan MG, Goeddel DV. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNFreceptor 1 signal transduction pathways. Cell. 1996;84:299-308
35. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell. 1995;81:505-512
36. Hsu H, Xiong J, Goeddel DV. The TNF receptor 1 associated protein TRADD signals cell death and NFκB activation. Cell. 1995;81:495-504

37. Liu Z, Hsu H, Goeddel DV, Karin M. Dissection of TNFreceptor 1 effector functions: JNK activation is not linked to apoptosis while NFκB activation prevents cell death. Cell. 1996;87:565-576
38. Stanger BZ, Leder P, Lee TH, Kim E, Seed B. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell. 1995;81:513-523
39. Hsu H, Huang J, Baichwal V, Goeddel DV. TNFdependent recruitment of the protein kinase RIP to the TNFreceptor-1 signaling complex. Immunity. 1996;4:387-396
40. Rothe M, Wong SC, Henzel WJ, Goeddel DV. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell. 1994;78:681-692
41. Baeuerle PA, Henkel T. Function and activation of NFκB in the immune system. Annu Rev Immunol. 1994;12:141-179
42. Introna M, Mantovani A. Early activation signals in endothelial cells. Stimulation by cytokines. Arterioscler Thromb Vasc Biol 1997;17:423-428
43. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T. The ubiquitin-proteasome pathway is required for processing the NFκB1 precursor protein and the activation of NFκB. Cell. 1994;78:7773-7785
44. Malinin NL, Boldin MP, Kovalenko AV, Wallach D. MAP3K-related kinase involved in NFκB induction by TNF CD95, IL-1. Nature. 1997;385:540-544
45. Régnier CH, Song HY, Gao X, Goeddel DV, Cao Z, Rothe M. Identification and characterization of an IκB kinase. Cell. 1997;90:373-383
46. Song HY, Rothe M, Goeddel DV. The tumor necrosis factor inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NFκB activation. PNAS 1996;93:6721-6725
47. Krikos A, Laherty CD, Dixit VM. Transcriptional activation of the tumor necrosis factor-inducible zinc finger protein, A20, is mediated by κB elements. 1992;267:17971-17976
48. Schütze S, Potthof K, Machleidt T, Berkovic D, Wiegmann K, Krönke M. TNF activates NFκB by phosphatidylcholine-specific phospholipase C-induced 'acidic' sphingomyelin breakdown. Cell. 1992;71:765-776
49. Weber C, Erl W, Pietsch A, Danesch U, Weber PC. Docosahexaenoic acid selectively attenuates induction of vascular cell adhesion molecule-1 and subsequent monocytic cell adhesion to human endothelial cells stimualted by tumor necrosis factor-α. Arterioscler Thromb Vasc Biol 1995;15:622-628
50. Modur V, Zimmerman GA, Prescott SM, McIntyre TM. Endothelial cell inflammatory responses to tumor necrosis factor α: ceramide-dependent and – independent mitogen activated protein kinase cascades. J Biol Chem 1996;271:13094-13102
51. Adam-Klages S, Adam D, Wiegmann K, Struve D, Kolanus W, Schneider-Mergener J, Krönke M. FAN, a novel WD-repeat protein, couples the p55 TNFreceptor to neutral sphingomyelinase. Cell. 1996;86:937-947
52. Wiegman, Schütze S, Machleidt T, Witte D, Krönke M. Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. Cell. 1994;78:1005-1015

53. Kolesnick R, Golde DW. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signalling. Cell. 1994;77:325-328
54. Raines MA, Kolesnick RN, Golde DW. Sphingomyelinase and ceramide activate mitogen activated protein kinase in myeloid HL-60 cells. J Biol Chem. 1993;268:14572-14575
55. L'Allemain G. Deciphering the MAP kinase pathway. Progress in Growth Factor Research. 1994;5:291-334
56. Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell. 1995;80:179-185
57. Cano E, Mahadevan LC. Parallel signal processing among mammalian MAPKs. Trends in Biol Sci. 1995;20:117-122
58. Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvater SW, Strickler JE, McLaughlin MM, Siemens IR, Fisher SM, Livi GP, White JR, Adams JL, Young PR. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature. 1994;372:739-746
59. Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, Davis RJ. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 1995;270:7420-7426
60. Read MA, Whitley MZ, Gupta S, Pierce JW, Best J, Davis RJ, Collins T. Tumor necrosis factor α-induced E-selectin expression is activated by the nuclear factor-κB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways. J Biol Chem 1997;272:2753-2761
61. Cybulsky MI, Fries JWU, Williams AJ, Sultan P, Davis VM, Gimbrone MA, Collins TA. Alternative splicing of human VCAM-1 in activated vascular endothelium. Am J Pathol 1991;138:815-820
62. Neish AS, Williams AJ, Palmer HJ, Whitley MZ, Collins T. Functional analysis of the human vascular cell adhesion molecule-1 promotor. J Exp Med. 1992;176:1583-1593
63. Iademarco MF, McQuillan JJ, Rosen GD, Dean DC. Characterization of the promotor for vascular cell adehsion molecule-1 (VCAM-1). J Biol Chem. 1992;267:16323-16329
64. Orkin SH. Globin gene regulation and switching: circa 1990. Cell. 1990;63:665-672.
65. Neish AS, Read MA, Thanos D, Pine R, Maniatis T, Collins T. Endothelial interferon regulatory factor-1 cooperates with NFκB as a transcriptional activator of vascular cell adhesion molecule 1. Mol Cell Biol. 1995;15:2558-2569
66. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NFκB and cytokine inducible enhancers. FASEB J. 1995;9:899-909
67. Marui N, Offerman MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, Medford RM. Vascular cell adehsion molecule-1 gene transcription and expression are regulated through an anti-oxidant sensitive mechanism in human vascular endothelial cells. J Clin Invest 1993;92:1866-1874
68. Borek C. The pro-oxidant state in initiation and promotion by carcinogens and modalities used in cancer therapy and the anticarcinogenic action of antioxidants in: Superoxide and SOD in

Chemistry Biology and Medicine, Proc. 4th Int. Conf edited by G. Rotilio, Elsevier Amsterdam 1986:451-457

69. Saran M, Bors W. Oxygen radicals acting as chemical messengers: a hypothesis. Free Rad Res Commun. 1989;7:213-220
70. Demple B, Amabile-Cuevas CF. Redox-redux: The control of oxidative stress response. Cell. 1991;67:837-839
71. Farr SB, Kogoma T. Oxidative stress responses in Escherichia coli and Salmonella typhimurium. Microbiological reviews. 1991;55:561-585
72. Amabile-Cuevas CF, Demple B. Molecular characterization of the soxRS genes of Escherichia coli: Two genes control a superoxide stress regulon. Nucleic Acids Research. 1991;19:4479-4484
73. Storz G, Tartaglia LA, Ames BN. Transcriptional regulation of oxidative stress-inducible genes: Direct activation by oxidation. Science. 1990;248:189-194
74. Schreck R, Meier B, Männel DN, Dröge W, Baeuerle PA. Dithiocarbamates as potent inhibitors of NFκB activation in intact cells. J Exp Med. 1992;175:1181-1194
75. Schreck R, Alberman K, Baeuerle PA. Nuclear factor κB: an oxidative stress responsive factor of eukaryotic cells. Free Rad Res Commun. 1992;17:221-237
76. Huot J, Houle F, Marceau F, Landry J. Oxidative stress-induced actin reorganization mediated by the p38 mitogen-activated protein kinase/ heat shock protein 27 pathway in vascular endothelial cells. Cir Res 1997;80:383-39

6 Evidence against the involvement of multiple radical generating sites in the expression of VCAM-1

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ABSTRACT

The present study was undertaken to investigate the hypothesis that multiple oxygen radical generating systems contribute to the tumor necrosis factor α (TNF)-stimulated transcriptional activation of the vascular cell adhesion molecule (VCAM)-1 in endothelial cells. Experimental evidence has implicated the cytochrome P450 monooxygenase and a phagocyte type NADPH-oxidase as a source of oxygen radicals in these cells. We show here that endothelial cells exhibit cytochrome P450 activity by measuring the O-dealkylation of the exogenous substrate 7-ethoxyresorufin, but components of the phagocyte-type NADPH oxidase could not be demonstrated in endothelial cells. In that latter respect it was surprising that the NADPH oxidase inhibitor apocynin completely prevented the accumulation of VCAM-1 mRNA. However, we found that apocynin also acts as an inhibitor of cytochrome P450 activity in endothelial cells. Therefore the inhibitory effect of apocynin on the induction of VCAM-1 may no longer be used to demonstrate a role for the NADPH oxidase in this process. Furthermore, different cytochrome P450 inhibitors Co^{2+}, metyrapone, SKF525a decreased the endothelial VCAM-1 expression stimulated by TNF. Also under hypoxic conditions the expression of VCAM-1 was reduced. On this basis we assume that the oxygen dependent step in the intracellular signalling cascade underlying the TNF stimulated transcriptional activation of VCAM-1 resides in the activity of a cytochrome P450 dependent monooxygenase. The finding that the phospholipase A_2 inhibitor bromophenacylbromide inhibited the expression of VCAM-1 may indicate that arachidonic acid serves as a substrate for the cytochrome P450 monooxygenase reaction, but further research is needed to elucidate the particular cytochrome P450 family member mediating the expression of VCAM-1.

INTRODUCTION

Endothelial cell adhesion molecules play an important role in the recruitment of leukocytes into the extravascular space during the inflammatory response and in the repair of tissue injury (1, 2). The expression of the adhesion molecules vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1) and endothelial leucocyte adhesion molecule-1 (E-selectin) is induced (VCAM-1 and E-selectin) or increased (ICAM-1) by the cytokine tumor necrosis factor-α (TNF) (1, 2). A pivotal event in the stimulated transcription of the adhesion molecule genes is the activation of the transcription factor nuclear factor-κB (NFκB), a family of dimeric transcription factor complexes. NFκB resides in the cytosol in an inactive form complexed to members of the inhibitory protein family IκB. Upon activation IκB is removed from the complex by degradation (IκB-α) or processing (p105) in the proteasome proteolytic pathway (3, 4, 5). NFκB then translocates to the nucleus where it binds to κB regulatory elements in the promoter regions of the VCAM-1, ICAM-1 and E-selectin genes (6, 7, 8). Although essential, the activation of NFκB is not sufficient for cytokine-induced transcription. Several other factors are likely involved in the assembly of unique transcriptional activation complexes (5).

The signal transduction pathways underlying the expression of VCAM-1, ICAM-1 and E-selectin may not be completely overlapping. Marui *et al.* showed that the expression of VCAM-1, but not ICAM-1, is controlled by the redox status of the endothelial cell (9). The redox status supposedly is modulated through the formation of reactive oxygen species (ROS) on the one hand, and the level of intracellular antioxidants and the activity of the antioxidant enzymes on the other. It has been hypothesized that ROS function as second messenger molecules in the activation of NFκB (10).

Previously, it has been suggested that in L929 fibroblast cells the mitochondrion is the source of oxygen radicals which would serve as common mediators of the cytotoxic and gene regulatory effects of TNF in these cells (11).

The TNF-induced expression of VCAM-1 seems to involve several radical-generating systems. It has been suggested by Weber *et al.* that in endothelial cells these systems include the flavoenzymes cytochrome P450 monooxygenase and a phagocyte-type NADPH oxidase, since inhibition of these two systems by, respectively, SKF525a and apocynin (4-hydroxy,-3-methoxy-acetophenone) inhibited the TNF induced expression of VCAM-1 specifically (12). This effect most likely was due to an effect on the activation of NFκB, as demonstrated for

SKF525a (12). However, in contrast to the flavoprotein dependent cytochrome P450 monooxygenase (13) the expression of a phagocyte-type NADPH-oxidase activity remains to be demonstrated in endothelial cells (12). Leaving the possibility that the effect of apocynin on the endothelial VCAM-1 induction has to be attributed to a completely different mode of action.

It has been demonstrated before that apocynin can inhibit cytochrome P45011β in sheep adrenal cells (14). This might imply that apocynin, like SKF525a, inhibits the cytochrome P450 monooxygenase present in endothelial cells. This would shed doubt on the idea that multiple radical generating sites are involved in the TNF-induced expression of VCAM-1. We therefore questioned whether the flavoenzyme cytochrome P450 monooxygenase may be the sole oxygen dependent step in the intracellular signalling cascade leading to the expression of VCAM-1. To that end we have investigated the presence of NADPH oxidase and questioned whether apocynin could act as an inhibitor of cytochrome P450 monooxygenase in endothelial cells. Finally, we studied the effect of hypoxia and inhibition of the cytochrome P450 electron transport chain on the expression of VCAM-1.

MATERIALS AND METHODS

Monoclonal Antibodies

Monoclonal antibody (mAb) clone MEM112 directed against ICAM-1, and mAb clone 1G11B1 directed against VCAM-1, and clone ENA1 directed against E-selectin were obtained from Monosan (Uden, The Netherlands). Mouse IgG1κ, was purchased from Sigma (St. Louis, MO) and fluorescein isothiocyanate (FITC)-conjugated rabbit-anti-mouse Fab_2 fragment STAR9 from Serotec (Oxford, UK). Antisera and mAb's directed respectively against p47-*phox*, p67-*phox* and gp91-*phox* were a generous gift of dr. A. J. Verhoeven (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).

Reagents

Reagents were obtained from Merck (Darmstadt, Germany) unless specified otherwise. Diphenylene iodonium obtained from Sigma was dissolved in dimethylsulfoxide by sonication as a 20 mmol/L stock.

Culture and FACS Analysis of Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were cultured as previously described (15) according to the method described by Jaffe *et al.* (16) in M199 (Flow Laboratories) supplemented with 10% newborn calf serum, 10% human serum kindly provided by the Red Cross Bloodbank, Rotterdam, The Netherlands, endothelial cell growth factor, 15 U/ml heparin, 50 U/ml penicillin, and 5 μg/ml streptomycin, under 95% air- 5 % CO_2. Confluent endothelial monolayers were treated as indicated in the legends. Cells were detached and incubated for 30 min at 4°C with either monoclonal antibody clone IG11B1 (Monosan, Uden, The Netherlands) directed against VCAM-1, or clone MEM112 (Monosan) directed against ICAM-1, or clone ENA1 directed against E-selectin, or control mouse IgG1κ as described before (15). The second antibody was a fluorescein isothiocyanate conjugated rabbit anti-mouse Fab_2 fragment. Membrane antigen expression was analysed by fluorescence activated cell sorting (FACS) as previously described (17), and is expressed as median linear fluorescence intensity (F.I.). Data were corrected for background fluorescence due to binding of IgG1κ, unless specified otherwise.

Assay of Cytochrome P450 Activity in Endothelial Cells

The cytochrome P450 activities were assessed using intact cells according to the method described in detail by Donato *et al* (18). TNF-stimulated endothelial cells (25 cm^2) were incubated with 7-ethoxyresorufin (10 µmol/L, Sigma) which can be dealkylated by cytochrome P5450IA1 (CYPIA1) (18,19) and by cytochrome P450-AA (20). In addition to this cells were also incubated with 7-pentoxyresorufin (20 µmol/L, Sigma) which is specifically dealkylated by P450IIB1 (18,19). After 4 h at 37°C aliquots of supernatant culture media were incubated in the presence of 150 Fishman units of β-glucuronidase/ ml and 1200 Roy units of arylsulfatase/ ml (Boehringer, Mannheim, Germany) for 2 h at 37°C. The fluorescence of resorufin was measured in a Perkin Elmer fluorimeter with excitation and emission wavelengths set at 530 nm and 590 nm, respectively. A standard curve of resorufin (Sigma) was prepared in culture medium. The results are expressed in pmol resorufin/ mg cell protein.

Immunoblot Analysis

Neutrophilic granulocytes (granulocytes) were isolated as previously described (15) and lysed overnight at 4°C in 10 mol/L HEPES, 0.25 mol/L, 5 mmol/L EDTA, 100 µg/ml leupeptin, 2 mmol/L PMSF, and 10 mmol/L β-mercaptoethanol, pH7.5. Endothelial cells were lysed overnight at 4°C in 4 mmol/L EDTA, 50 mmol/L Tris, 150 mmol/L NaCl, 0.5% (v/v) nonidet P40, and 1 mmol/L PMSF, pH 7.4. Twenty µg of cell homogenate protein was analysed by SDS PAGE. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride membrane (Boehringer Mannheim, Germany). Membranes were blocked for 1h with 3% (w/v) albumin in Tris-buffered saline with 0.1% (v/v) TWEEN (TTBS). Blots were washed with TTBS and incubated for 18 h with 1:2000 rabbit antiserum directed against the C-terminus of p67-*phox*, or 1:2000 rabbit antiserum against the C-terminus of p47-*phox*, or 1:1000 mouse mAb 43 directed against gp91-*phox* in TTBS, or in TTBS without antiserum to control for binding of the secondary antibody. Next, immunoblots were incubated with peroxidase conjugated goat-anti-rabbit mAb (1:5000) or with peroxidase conjugated goat-anti-mouse mAb (1:10000). Blots were developed with ECL (Amersham, UK).

Hypoxic Incubations

Hypoxic incubations were performed as previously described at 37°C (15). In the incubator a continuous flow of 95% N_2-5% CO_2 humidified and warmed to 37°C was maintained during hypoxic incubations. Control incubations were done at 20% O_2-75% N_2-5% CO_2 at 37°C. Buffers and media were equilibrated to ambient conditions before starting the experiment.

Polymerase Chain Reaction

Total RNA was isolated from $2*10^6$ HUVEC stimulated with TNF (0 - 100 U/ml in complete M199) according to the method of Chomszynski *et al.* (21). cDNA was produced from 75 ng of each RNA extract by M-MLV reverse transcriptase (Promega) as described before (21). For VCAM-1 specific oligonucleotide primers were used (forward primer: 5'-CGGGATCCATCCACAAAGCTGCAAGAA-3', and reverse primer: 5'-GCGAATTCGCCACCACTCATCTCGATTT-3') which have been previously described (22). For ICAM-1 specific oligonucleotide primers were used described by Saito *et al.* (forward primer 5'-TGACCATCTACAGCTTTCCGGC-3' and reverse primer 5'-AGCCTGGCACATTGGAGTCTG-3') (23). cDNA was amplified by 30 cycles with Goldstar DNA polymerase (Eurogentec) in a Perkin Elmer thermocycler 480 with the annealing temperature set at 55°C or 60°C, respectively for VCAM-1 and ICAM-1. Homology of the PCR product with the VCAM-1 cDNA or the ICAM-1 cDNA was confirmed by sequence analysis.

Northern Blot Analysis

Total cellular RNA (20 µg) was size-fractioned using 1.2 % agarose formaldehyde gel in the presence of 1 µg ethidium bromide. The RNA was transferred onto Hybond and covalently linked by ultraviolet irradiation. Hybridizations were performed at 42 °C for 18 h in 50% deionized formamide, 1 mol/L NaCl, 2.5 % dextran sulfate, 35 µg/ml denatured fish DNA in 20% P buffer (1% BSA, 1% polyvinylpyrrolidone, 1% Ficoll, 250 mmol/L Tris-HCl, 0.5% Na-pyrophosphate, 5% SDS). The PCR product (approximately 50 ng cDNA) was radiolabeled with [α-^{32}P]dCTP (±50 µCi per hybridization) by the use of a labelling kit (Amersham). After hybridization, filters were washed with a final stringency of 0.2 x SSC (1 x SSC = 150 mmol/L NaCl, 15 mmol/L Na citrate), 0.1% SDS at room temperature. Autoradiograms were quantified by phosphor-imaging (Molecular Imaging System GS-363, Biorad).

Statistical Analysis

Data are expressed as the means ± standard error of mean (SE). The differences between means was assessed by Student's *t*-test. Significance was determined at the 95% confidence level.

RESULTS

Cytochrome P450 Activity in Endothelial Cells

To assess whether apocynin inhibits cytochrome P450 dependent monooxygenase activity in TNF-stimulated endothelial cells, we set out to determine the rate of dealkylation of the cytochrome P450 substrates 7-ethoxyresorufin and 7-pentoxyresorufin in unstimulated and TNF-stimulated endothelial cells. The basal rate of dealkylation of 7-ethoxyresorufin was 74 $pmol.mg^{-1}$ cell protein in 4 h. Stimulating the cells with 100 U/ml TNF did not significantly increase this rate, i.e. 101 $pmol.mg^{-1}$ in 4 h in the presence of TNF.

Endothelial cells did not dealkylate 7-pentoxyresorufin within 4 h of incubation. Indicating the absence of the cytochrome P450 family member CYPIIB1. Even with an extended incubation time of 18 h we did not detect any dealkylation of 7-pentoxyresorufin. Stimulating the endothelial cells with TNF also did not reveal any significant CYPIIB1 activity (data not shown).

Effect of Apocynin and Diphenylene Iodonium on the Cytochrome P450 Enzyme Activity

The effect of apocynin on the cytochrome P450 dependent ethoxyresorufin dealkylation by endothelial cells stimulated with TNF was investigated next. Cells were preincubated with apocynin or vehicle for 30 min, next 7-ethoxyresorufin dealkylation was assessed in the presence or absence of apocynin. The results presented in Fig. 1 show that apocynin significantly decreased the dealkylation of 7-ethoxyresorufin by TNF-stimulated endothelial cells to about 50%, i.e., 52 $pmol.mg^{-1}$ in 4 h ($P<0.05$). In unstimulated endothelial cells apocynin decreased the dealkylation rate from 74 $pmol.mg^{-1}$ to 31 $pmol.mg^{-1}$ in 4 h ($P<0.05$). As a control we also assessed the effect of inhibition of the NADPH cytochrome P450 reductase, essential for cytochrome P450 activity, by the well known flavoprotein inhibitor diphenylene iodonium (DPI). The results presented in Fig 1 show that DPI completely inhibited the dealkylation of 7-ethoxyresorufin in endothelial cells.

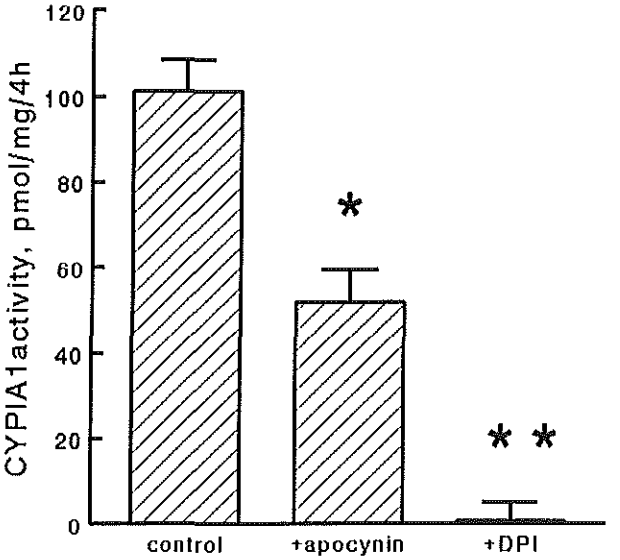


Fig 1. Effect of apocynin and diphenylene iodonium (DPI) on the cytochrome P450 activity in endothelial cells. Endothelial cells were preincubated with apocynin (600 µmol/L) or diphenylene iodonium (20 µmol/L) for 30 min, next the cells were stimulated with TNF (100 U/ml) and 7-ethoxyresorufin dealkylation was assessed in the presence of apocynin or DPI. The results represent at least three experiments. Asterisks denote significant difference from control (*; P<0.05 and **; P(0.01).

Assessment of the NADPH Oxidase Components P47-phox, P67-phox and gp91-phox in Endothelial Cells

To assess whether endothelial cells express the phagocyte-type NADPH oxidase, homogenates of resting or TNF-stimulated endothelial cells were analysed by immunoblotting, and the expression of the NADPH oxidase components p47-*phox*, p67-*phox* and gp91-*phox* were compared with equal amounts (20 µg) of homogenate protein of resting or phorbol myristate acetate (PMA) stimulated granulocytes. The results presented in Fig 2 show that endothelial cells do not express p47-*phox*, p67-*phox* and gp91-*phox* either in the absence or presence of TNF. Whereas in granulocytes the p47-*phox* and p67-*phox* are amply present either in resting or PMA-treated cells. Gp91-*phox* could also be detected but to a lesser extent in resting and stimulated granulocytes (Fig 2).

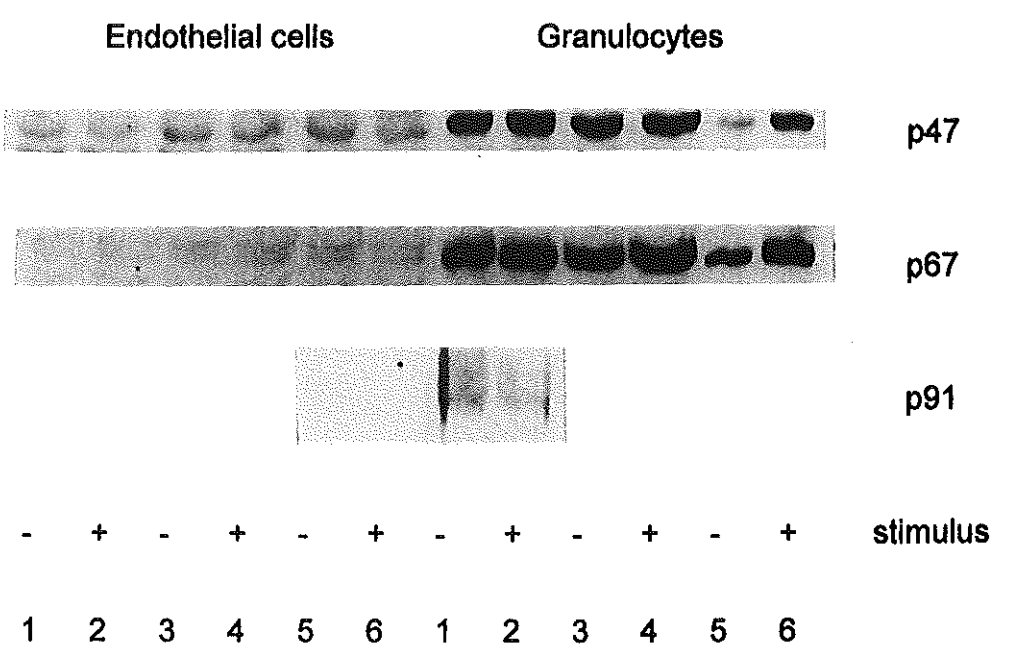


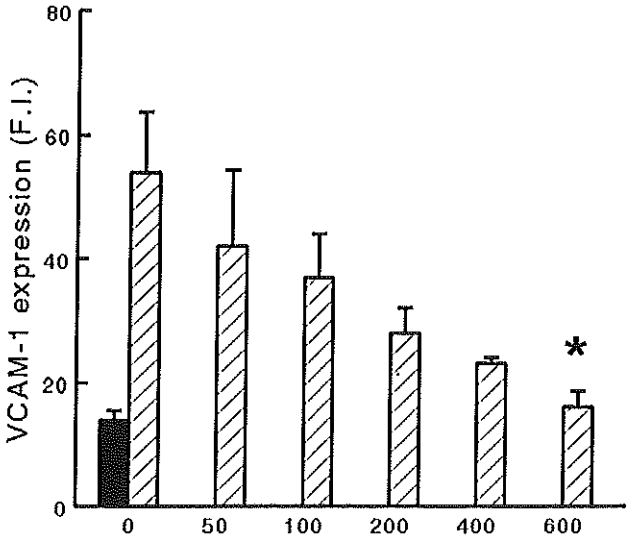
Fig 2. Assessment of NADPH oxidase components p47-*phox*, p67-*phox* and gp91-*phox* in endothelial cells and granulocytes. Equal amounts of endothelial cell en granulocyte homogenates (20 µg of protein) were analyzed by SDS-PAGE and immunoblotting as described. Endothelial cells were stimulated in three separate experiments with 0 or 100 U/ml TNF (1-6) for 4 h. Granulocytes were treated with 0 or 100 ng/ml phorbol myristate acetate in three separate experiments (1-6). The NADPH components p47-*phox*, p67-*phox*, and gp91-*phox* are respectively denoted as p47, p67 and p91. The density of the bands in the endothelial cell lanes was similar to that obtained when blots were incubated with only the secondary antibody (data not shown).

Effect of Apocynin and DPI on the Expression of VCAM-1

To confirm the inhibitory effect of apocynin on VCAM-1 protein expression demonstrated before (12) endothelial cells were incubated with increasing concentrations of apocynin for 30 min to 1 h. Next, the cells were stimulated with 100 U/ml of TNF in the presence or absence of apocynin. The expression of VCAM-1 protein (Fig 3a) was inhibited by apocynin in a dose dependent manner, with a maximum effect at 600 µmol/L. At this concentration apocynin almost completely inhibited the accumulation of VCAM-1 mRNA (Fig 3b).

This effect was specific for VCAM-1 since the accumulation of ICAM-1 protein and mRNA were not significantly affected by apocynin (Fig 4a and b).

Fig 3 a



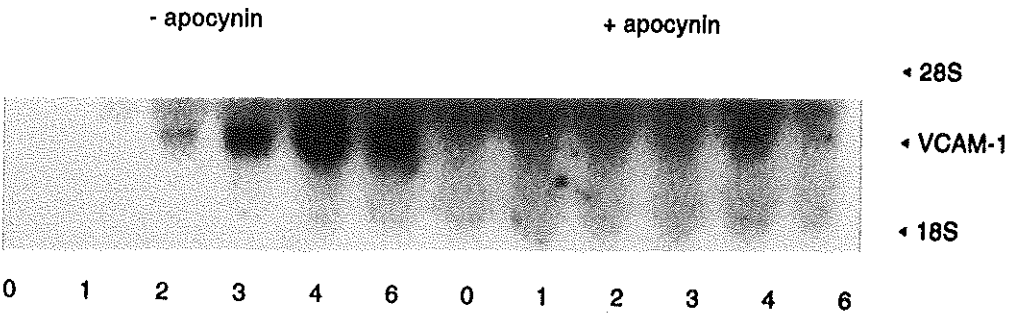


Fig 3 b

Fig 3 a+b Effect of apocynin on the TNF induced expression of VCAM-1 protein (3a) and mRNA (3b). Endothelial cells were preincubated with apocynin (0 - 600 μmol/L). Next, the cells were stimulated with TNF (100 U/ml) in the presence of apocynin. After 6 h of stimulation with TNF the expression of VCAM-1 protein was assessed by FACS analysis (hatched bars, Fig 3a). The black bar represents binding of anti-VCAM-1 mAb to unstimulated endothelial cells, background fluorescence of cells incubated with IgG1κ, i.e., 5.4 F.I. ± sd= 1.1, remained unchanged under the applied conditions. In Fig 3b the TNF stimulated accumulation of VCAM-1 mRNA in time was assessed in the presence or absence of apocynin (600 μmol/L) by Northern blotting.

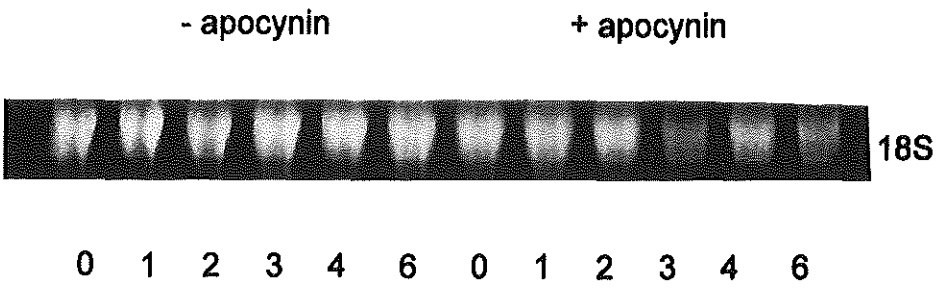


Fig 3c The ethidium bromide stained 18S RNA band of each lane depicted in Fig 3b is shown as a control for equal loading and quality of the RNA samples. Results represent at least three experiments.

Fig 4a

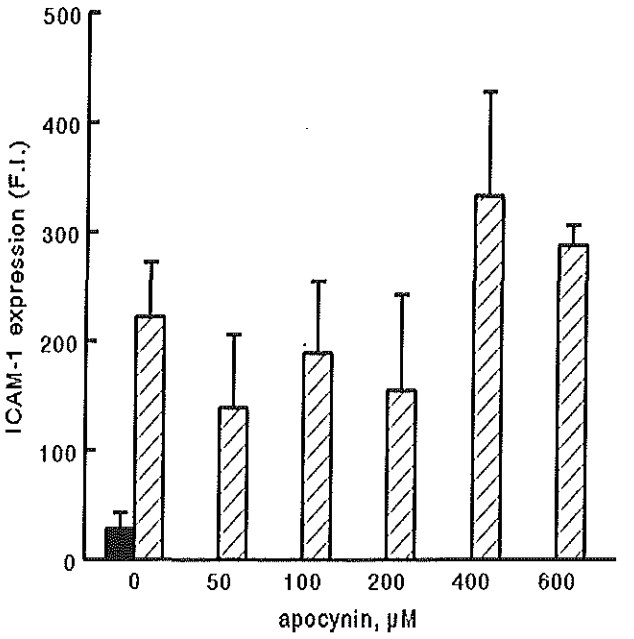


Fig 4b

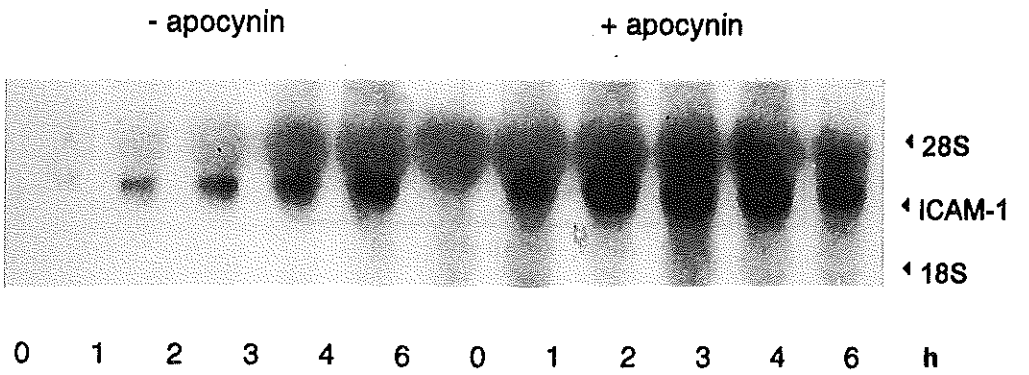


Fig 4. Effect of apocynin on the TNF induced expression of ICAM-1 protein (4a) and mRNA (4b). Endothelial cells were preincubated with apocynin (0 - 600 µmol/L). Next, the cells were stimulated with TNF (100 U/ml) in the presence of apocynin. After 6 h of stimulation with TNF the expression of ICAM-1 protein was assessed by FACS analysis (hatched bars, Fig 4a). The black bar represents aspecific binding of anti-ICAM-1 mAb to unstimulated endothelial cells, background fluorescence was 5.4 F.I. ± sd= 1.1, and remained unchanged under the applied conditions. In Fig 4b the TNF stimulated accumulation of ICAM-1 mRNA in time was assessed in the presence or absence of apocynin (600 µmol/L) by Northern blot. In Fig 3c the ethidium bromide stained 18S RNA band of each lane is shown as a control for equal loading and quality of the RNA samples. Results represent at least three experiments.
experiments.

To further substantiate the involvement of flavoenzymes in the expression of VCAM-1 endothelial cells were treated with increasing concentrations of DPI (0, 10 and 20 µmol/L) or vehicle, and next stimulated with 100 U/ml TNF in the presence or absence of DPI. The results presented in Fig. 5 show that 20 µmol/L of DPI significantly inhibited VCAM-1 expression. The equivalent concentration of the vehicle DMSO had no effect on the expression of VCAM-1, which amounted to 94% of the control. The inhibitory effect of DPI was specific for the expression of VCAM-1 since the expression of ICAM-1 remained unaffected by DPI up to the maximum concentration of 20 µmol/L (Fig 5).

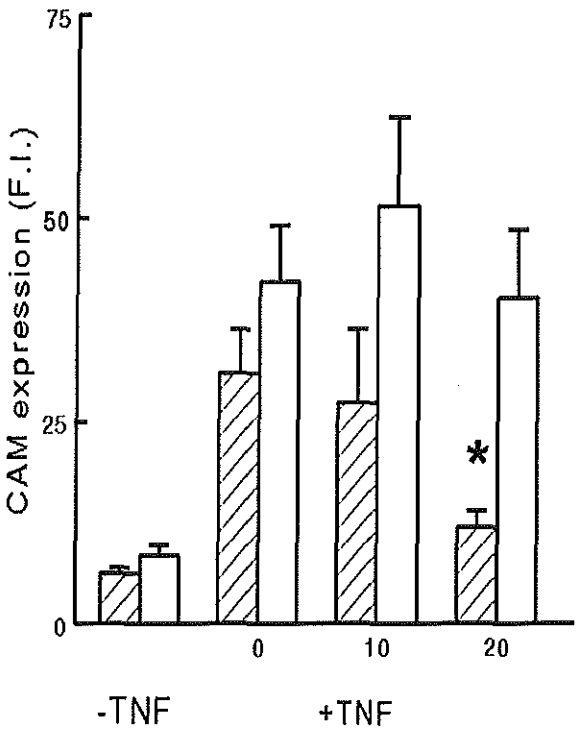


Fig 5. Effect of diphenylene iodonium (DPI) on the expression VCAM-1 and ICAM-1. Endothelial cells were preincubated with increasing concentrations of DPI (0 - 20 µmol/L), and subsequently stimulated with TNF (100 U/ml) in the presence or absence of DPI. After 6 h of stimulation the expression of VCAM-1 (hatched bars) or ICAM-1 (white bars) was assessed by FACS analysis. Background fluorescence of cells incubated with IgG1κ was 5 F.I. and did not change under the applied conditions. Results represent three experiments. * Significant difference from control ($P<0.05$). CAM; Cell Adhesion Molecule.

To exclude the possibility that the effect of DPI could be attributed to an effect on the mitochondrial NADH dehydrogenase (or NADH Q reductase) we tested the effect of rotenone on the endothelial expression of VCAM-1. After treatment of endothelial cells with 5 µmol/L

rotenone their oxygen consumption measured by a Clark type electrode was inhibited by 70%. At this concentration rotenone did not inhibit the TNF stimulated endothelial VCAM-1 expression. In fact it seemed to increase VCAM-1 expression from 22 F.I. (n=2, range 20-23) to 51 F.I. (n=2, range 46-55). This effect was not further investigated since it was beyond the scope of the present study.

Effect of Cytochrome P450- and Phospholipase A_2 Inhibitors on VCAM-1 Expression

To establish the role of the cytochrome P450 monooxygenase in the TNF-stimulated expression of VCAM-1 we used different inhibitors of the cytochrome P450 electron transport system and tested their effect on the expression of VCAM-1 and ICAM-1 by TNF-stimulated endothelial cells. The results presented in Table 1 show that the majority of the cytochrome P450 inhibitors used in this study (significantly) decreased the TNF stimulated expression of VCAM-1. The most dramatic decrease in VCAM-1 expression was observed for Co^{2+}, a metal which is known to decrease the intracellular content of cytochrome P450. The effect of Co^{2+} was only observed with a preincubation time of 18 h and not with a shorter preincubation time of 30 min (data not shown). In contrast to Co^{2+}, metyrapone and SKF525a, another well known cytochrome P450 inhibitor allylisopropylacetamide (AIA), which causes breakdown of the cytochrome P450 haem by a process called 'suicidal inactivation', did not at all affect the endothelial VCAM-1 expression. None of the inhibitors used in this study significantly decreased the TNF-stimulated expression of ICAM-1.

Inhibition of phospholipase A_2 activity by bromophenacylbromide (BPB) resulted in a 60-70 % decrease ($P<0.05$) of VCAM-1 expression in TNF stimulated endothelial cells. Whereas the expression of ICAM-1 remained unaffected (Table 1).

TABLE 1. Effect of cytochromeP450 and phospholipase A_2 inhibition on the expression of VCAM-1 by TNF stimulated endothelial cells.

Inhibitor	Conc.	% VCAM-1 expression	P-value	% ICAM-1 expression	P-value
Cytochrome P450 Inhibitor					
Co^{2+}	0.2 mM	17 (n=3)[a]	0.015	82 (n=3)[a]	n.s.
AIA	0.2 mM	109 (n=3)[a]	n.s.	100 (n=3)[a]	n.s.
Metyrapone	1.0 mM	32 (n=3)[b]	0.017	83 (n=3)[b]	n.s.
SKF525a	0.1 mM	48 (n=3)[b]	0.041	89 (n=3)[b]	n.s.
Phospholipase A_2 Inhibitor					
BPB	10 μM	34 (n=4)[b]	0.048	91 (n=2)[b]	

Cells were pretreated with inhibitor prior to stimulation with TNFα in the presence of inhibitor:

[a] for 18 h

[b] for 30 min -1 h

AIA: allylisopropylacetamide

BPB: bromophenacylbromide

Effect of Hypoxia on the Expression of VCAM-1

To investigate whether the TNF-stimulated induction of VCAM-1 by endothelial cells depends on the presence of oxygen, we studied the effect of hypoxia on the surface expression of VCAM-1. Endothelial cells were preincubated under hypoxic conditions for 30 min, and next stimulated with increasing concentrations of TNF for 6 h under continuous hypoxia. The results presented in Table 2 show that hypoxia significantly decreased the TNF stimulated expression of VCAM-1 to about 60% of the normoxic control ($P<0.05$). To determine whether this effect was specific for the expression of VCAM-1 we also studied the effect of hypoxia on the expression of ICAM-1 at 6 h after stimulation with TNF, and of E-selectin at 4 h after stimulation, at which time E-selectin expression is maximal. The results show that the expression of both ICAM-1 and E-selectin under hypoxic conditions did not differ from the normoxic control (Table 2).

Table 2. Effect of hypoxia on the expression of the endothelial leucocyte adhesion molecules

Adhesion molecule	Normoxia	Hypoxia	P-value
VCAM-1	100%	57±14 % (n=4)	0.029
ICAM-1	100%	100% (n=2)	
E-selectin	100%	116% (n=2)	

Endothelial cells were kept under hypoxic or normoxic conditions for 30 min and next stimulated with 100 U/ml TNF for 6 h (VCAM-1 and ICAM-1) or 4 h (E-selectin), while kept under the same ambient conditions. Data are presented as percentage of the normoxic control. Statistical significance of the difference in fluorescence intensities was assessed by Student's t-test for paired data.

DISCUSSION

The major conclusion to be drawn from the present study is that the proposed involvement of various flavoenzymes in the TNF-induced expression of VCAM-1 can be traced back to the cytochrome P450 dependent monooxygenase in endothelial cells. Based upon the evidence obtained with different enzyme inhibitors at least two flavoenzyme dependent systems have been implied that positively affect the endothelial expression of VCAM-1. These are the phagocyte-type NADPH oxidase and the cytochrome P450 monooxygenase electron transport system (12). It has been hypothesized that both the NADPH-oxidase and the cytochrome P450 monooxygenase, which both can be a source of reactive oxygen species (ROS), mediate the transcriptional activation of VCAM-1 by the generation of these ROS (12). In our opinion the idea that these two systems contribute to a putative oxygen radical pool can no longer be sustained. While apocynin inhibits the NADPH oxidase in neutrophilic granulocytes (24), we now demonstrate that apocynin is an inhibitor of cytochrome P450 activity in endothelial cells. Furthermore we show here that endothelial cells do not express any of the NADPH oxidase components p47-*phox*, p67-*phox*, or gp91-*phox* either under resting or stimulated conditions. Apparently, endothelial cells do not express the phagocyte-type NADPH oxidase. Thus, the inhibitory effect of apocynin on the induction of VCAM-1 and its inhibitory effect on cytochrome P450 activity may now support a role for the cytochrome P450 monooxygenase in the TNF-induced endothelial expression of VCAM-1. In the present study we also show that the TNF-stimulated expression of VCAM-1, but not ICAM-1 or E-selectin, is reduced under hypoxic conditions. These findings are in accordance with those of Klein *et al.* (25), although they reported a slightly larger decrease in the TNF stimulated VCAM-1 expression (about 60% inhibition) under hypoxic conditions. It remains to be demonstrated whether anoxia can completely abolish TNF stimulated VCAM-1 expression. If we consider these data together with the fact that various cytochrome P450 inhibitors and the NADPH cytochrome P450 reductase inhibitor DPI can decrease the endothelial VCAM-1 expression stimulated by TNF we conclude that the oxygen dependent step in the intracellular signalling cascade underlying the TNF stimulated transcriptional activation of VCAM-1 resides in the activity of a cytochrome P450 dependent monooxygenase.

By the use of the exogenous substrate 7-ethoxyresorufin we were able to demonstrate cytochrome P450 activity in intact endothelial cells. This activity can be attributed to the cytochrome P450 family members cytochrome P450IA1 (18,19) and P450-AA (20). The dealkylation of 7-ethoxyresorufin may therefore confirm the previous observation by Overby

et al. who showed that CYPIA1 is present in endothelial cells at the protein and mRNA level (13). However, a physiological substrate for CYPIA1 has not yet been identified. In contrast cytochrome P450-AA oxidizes arachidonic acid into the biologically active epoxyeicosatrienoic acids (20, 26). Our data on the inhibitory activity of the phospholipase A_2 inhibitor BPB on VCAM-1 expression indeed indicate that arachidonic acid may be the substrate for the cytochrome P450 dependent monooxygenase reaction.

An unexpected finding of the present study was that the well known cytochrome P450 inhibitor allylisopropylacetamide (AIA) failed to decrease VCAM-1 expression. Although we expected that AIA like Co^{2+} would drastically reduce VCAM-1 expression this observation may be explained by the selectivity of AIA for distinct cytochrome P450 family members, i.e. pentobaribital induced forms of cytochrome P450 (27). For example the cytochrome P450 PB-4 also known as the *CYP2B1* gene product is particularly susceptible to suicidal inactivation by AIA (28). We therefore conclude that the difference in expression of cytochrome P450 family members between phenobarbital-induced rat hepatocytes and human endothelial cells (18) could make the endothelial cells less susceptible to treatment with AIA. In this respect it may be relevant to note that we already demonstrated no dealkylating activity of 7-pentoxyresorufin in endothelial cells, whereas phenobarbital induced rat hepatocytes can dealkylate this substrate (18).

During cytochrome P450 dependent monooxygenation electrons are transferred from NADPH to the cytochrome P450 heme. One electron is used to reduce the heme iron, and the second to subsequently bind and cleave the oxygen molecule to generate the active species for insertion into the substrate (29). The flavoenzyme NADPH-cytochrome P450 reductase temporarily stores electrons during this transfer. Iodonium compounds like DPI typically inhibit flavoenzymes that function as one-electron donors, accepting two electrons but passing them one singly (30). Whereas all known DPI *insensitive* flavoenzymes simultaneously transfer two electrons during catalysis (30). Therefore theoretically the inhibition of VCAM-1 induction could also have involved the iodonium sensitive flavoenzymes mitochondrial NADH dehydrogenase, xanthine oxidase, and nitric oxide (NO) synthase. An important role for the latter flavoenzyme has been suggested recently, when Khan *et al.* demonstrated that the increased delivery of NO suppresses the TNF-induced transcriptional activation of VCAM-1 (31). However, this negative regulatory role for the nitric oxide synthase can not explain the present observation that DPI inhibits the expression of VCAM-1, because then an increased expression of VCAM-1 would be anticipated. A role for the xanthine oxidase has been excluded previously by Weber *et al.* who demonstrated that allopurinol did not inhibit

VCAM-1 expression (12). In the present study we also excluded the possible contribution of the mitochondrial NADH dehydrogenase.

Taken together we postulate the cytochrome P450 monooxygenase as the sole oxygen dependent step in the TNF-stimulated induction of VCAM-1 by endothelial cells. A wide variety of structurally diverse compounds, e.g., steroids, fatty acids (including prostaglandins and leukotrienes) are metabolized by the cytochrome P450 monooxygenase system. Also the formation of the ROS hydrogen peroxide as a byproduct of the monooxygenation reaction has been described (32). Future research is needed to elucidate the type of cytochrome P450 isoenzyme and the nature of its product as the second messenger molecule in the expression of VCAM-1.

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REFERENCES

1. T.A. Springer (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*, 76, 301-314.
2. T.M. Carlos, J.M. Harlan (1994). Leukocyte-endothelial adhesion molecules. *Blood*, 84, 2068-2101.
3. V.J. Palombella, O.J. Rando, A.L. Goldberg, and T. Maniatis (1994). The ubiquitin-proteasome pathway is required for processing the NFκB1 precursor protein and the activation of NFκB. *Cell*, 78, 773-785.
4. E.B. Traenckner, S. Wilk, P.A. Baeuerle (1994). A proteasome inhibitor prevents activation of NFκB and stabilizes a newly phosphorylated form of IκB-α that is still bound to NFκB. *EMBO Journal*, 13, 5433-5441.
5. T. Collins, M.A. Read, A.S. Neish, M.Z. Whitley, D. Thanos, and T. Maniatis (1995). Transcriptional regulation of endothelial cell adhesion molecules: NFκB cytokine-inducible enhancers. *FASEB Journal*, 9, 899-909.
6. A.S. Neish, A.J. Williams, H.J. Palmer, M.Z. Whitley, and T. Collins (1992). Functional analysis of the human vascular cell adhesion molecule-1 promotor. *Journal of Experimental Medicine*, 176, 1583-1593.
7. G. Voraberger, R. Schafer, and C. Stratowa (1991). Cloning of the human gene for intercellular adhesion molecule 1 and analysis of its 5' regulatory region. Induction by cytokines and phorbol ester. *Journal of Immunology*, 147, 2777-2786.
8. J. Whelan, P. Ghersa, R.H. Hooft van Huijsduijnen, J. Gray, G. Chandra, F. Talabot, and J.F. Delamarter (1991). An NFκB-like factor is essential but not sufficient for cytokine induction of endothelial leukocyte adhesion molecule 1 (ELAM-1) gene transcription. *Nucleic Acids Research*, 19, 2645-2653.
9. N. Marui, M.K. Offerman, R. Swerlick, C. Kunsch, C.A. Rosen, M. Ahmad, R.W. Alexander, and R.M. Medford (1993). Vascular cell adhesion molecule-1 gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *Journal of Clinical Investigation*, 92, 1866-1874.
10. R. Schreck, P. Rieber, and P.A. Baeuerle (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NFκB transcription factor and HIV-1. *EMBO Journal*, 10, 2247-2258.
11. K. Schulze-Osthof, R. Beyaert, V. Vandevoorde, G. Haegeman, and W. Fiers (1993). Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. *EMBO Journal*, 12, 3095-3104.
12. C. Weber, W. Erl, A. Pietsch, M. Ströbel, H.W. Ziegler-Heitbrock, and P.C. Weber (1994). Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-κB mobilization and induction of vascular cell adheson molecule-1 in endothelial cells stimulated to generate radicals. *Arteriosclerosis and Thrombosis*. 14, 1665-1673.

13. L.H. Overby, S. Nishio, A. Weir, G.T. Carver, C.G. Plopper, R.M. Philpot (1992). Distribution of cytochrome P450 IA1 and NADPH-cytochrome P450 reductase in lungs of rabbits treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin: ultrastructural immunolocalization and in situ hybridization. *Molecular Pharmacology.* 41, 1039-1046.
14. P. Swart, van der Merwe K.J., A.C. Swart, P.C. Todres, J.H.S. Hofmeyr (1993). Inhibition of cytochrome P450 11ß by some naturally occuring acetophenones and plant extracts from the shrub *salsola tuberculatioformis. Planta Medica.* 59, 139-143.
15. A. Pietersma, N. de Jong, J.F. Koster, and W. Sluiter (1994). Effect of hypoxia on the adherence of granulocytes to endothelial cells *in vitro.* American Journal of Physiology 39, H874-H879.
16. E.A. Jaffe, R.L. Nachman, C.G. Becker, and C.R. Minick (1973). Culture of human endothelial cells derived from human umbilical veins: identification by morphologic and immunologic criteria. *Journal of Clinical Investigation.* 52, 2745-2756.
17. A. Pietersma, M. Kofflard, L.E.A. de Wit, T. Stijnen, J.F. Koster, P.W. Serruys, and W. Sluiter (1995). Late lumen loss after coronary angioplasty is associated with the activation status of circulating phagocytes before treatment. *Circulation* 91, 1320-1325.
18. M.T. Donato, M.J. Goméz-Lechnón, J.V. Casell (1993). A microassay for measuring cytochrome P450IA1 and P450IIB1 activities in intact human and rat hepatocytes cultured on 96-wells plates. *Analytical Biochemistry.* 213, 29-33.
19. M.D. Burke, S. Thompson, C.R. Elcombe, J. Halpert, T. Haaparanta, R.T. Mayer (1985). Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P450. *Biochemical Pharmacology.* 34, 3337-3345.
20. M. Laniado-Schwartzman, K.L. Davis, J.C. McGiff, R.D. Levere, N.G. Abraham (1988). Purification and characterization of cyotchrome P450 dependent arachidonic acid epoxygenase from human liver. *Journal of Biological Chemistry* 263, 2536-2542.
21. P. Chomczynski, and N. Sacchi (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. *Analytical Biochemistry.* 162, 156-159.
22. A. Pietersma, B.C. Tilly, M. Gaestel, N. de Jong, J.C. Lee, J.F. Koster, W. Sluiter (1997). P38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. *Biochemical and Biophysical Research Communications.* 230, 44-48.
23. I.Saito, K. Terauchi, M. Shimuta, S. Nishiimura, K. Yoshino, T. Takeuchi, K. Tsubota, N. Miyasaka (1993). Expression of cell adhesion molecules in the salivary and lacrimal glands of Sjogren's syndrome. *Journal of Clinical and Laboratory Analysis.* 7, 180-187.
24. J. Stolk, T.J.N. Hiltermann, J.H. Dijkman, A.J. Verhoeven (1994). Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *American Journal of Respiratory Cell and Molecular Biology* 11, 95-102.
25. C.L. Klein, H. Kohler, F. Bittinger, M. Otto, I. Hermanns, C.J. Kirkpatrick (1995). Comparative studies on vascular endothelium in vitro 2. Hypoxia: Its influences on endothelial cell proliferation and expression of adhesion molecules. *Pathobiology* 63, 1-8.

26. F.A. Fitzpatrick, R.C. Murphy (1989). Cytochrome P-450 metabolism of arachidonic acid: formation and biological actions of epoxygenase-derived eicosanoids. *Pharmacological Reviews.* 40, 229-241.
27. M.B. Baird, L.S. Birnbaum, H.V. Samis, H.R. Massie, J.A. Zimmerman (1976). Allylisopropylacetamide preferentially interacts with the phenobarbital-inducible form of rat hepatic microsomal P-450. *Biochemical Pharmacology* 25, 2415-2417.
28. D.J. Waxman, and C. Walsh (1982). Phenobarbital-induced rat liver cytochrome-P450 purification and characterization of two closely related isozyme forms. *Journal of Biological Chemistry.* 257, 10446-10457.
29. R.T. Okita, and B.S. Siler Masters (1992). Biotransformations: The cytochromes P450. In *Biochemistry with clinical correlations.* (ed. T. Devlin) Wiley, New York, pp 981-997.
30. V.B. O' Donnell, G.C.M. Smith, O.T.G. Jones (1994). Involvement of phenyl radicals in iodonium compound inhibition of flavoenzymes. *Molecular Pharmacology* 46, 778-785.
31. B.V. Khan, D.G. Harrison, M.T. Olbrych, R.W. Alexander, R.M. Medford (1996). Nitric oxide regulates vascular cell adhesion molecule-1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America,* 93, 9114-9119.
32. I.I Karuzina, and A.I. Archakov (1994). The oxidative inactivation of cytochrome P450 in monooxygenase reactions. *Free Radicals in Biology and Medicine.*16, 73-97.

7 P38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcription level

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ABSTRACT

The cytokine tumor necrosis factor-α (TNF) was found to stimulate the p38 mitogen activated protein (MAP) kinase signalling cascade in human umbilical vein endothelial cells. TNF increased the activity of the p38 substrate MAP kinase-activated-protein (MAPKAP) kinase 2 and the subsequent phosphorylation of the small heat shock protein Hsp27 about two to three fold. This stimulation was blocked almost completely by the specific p38 MAP kinase inhibitor SB203580. This inhibitor also suppressed the TNF-induced surface expression of the endothelial adhesion molecule vascular cell adhesion molecule (VCAM)-1. In contrast, inhibition of p38 MAP kinase had no effect on the stimulated surface expression of the intercellular cell adhesion molecule (ICAM)-1. VCAM-1 mRNA accumulation induced by TNF was not affected by SB203580 suggesting that the p38 MAP kinase signalling cascade regulates the endothelial expression of VCAM-1 at the post-transcriptional level.

INTRODUCTION

Under conditions of cellular stress, such as heat shock, UV radiation and osmotic stimulation, as well as subsequent to cytokine treatment at least two members of the superfamily of mitogen-activated protein (MAP) kinases are rapidly activated. These enzymes, which are distinct from the extra-cellularly regulated kinase (Erk)-1 and Erk2 stimulated by growth factors, are p38 MAP kinase/ reactivating kinase (RK) and c-jun amino terminal kinase (JNK)/ stress-activated protein (SAP) kinase. Like Erk1/2 these enzymes are activated through a cascade involving several other kinases as well as small GTP binding proteins (For review see 1-3).

Recently, two homologues of p38 MAP kinase have been identified in human monocytes by the use of a class of pyridinyl imidazoles (4). These drugs inhibit the production of interleukin-1β and tumor necrosis factor α (TNF) by stimulated monocytes. The pyridinyl imidazole SB203580 specifically inhibits p38 MAP kinase (5), and the subsequent phosphorylation of its physiological substrate MAP kinase-activated-protein-kinase-2 (MAPKAP kinase-2). Activation of MAPKAP kinase-2 leads to increased phosphorylation of the small heat shock proteins (Hsp) Hsp25 and Hsp27 (6,7).

TNF has been demonstrated to stimulate MAPKAP-kinase 2 activity (8) and to increase the phosphorylation of small Hsp in mammalian cells (9). In endothelial cells TNF induces the expression of vascular cell adhesion molecule-1 (VCAM-1), and increases the expression of the intercellular cell adhesion molecule-1 (ICAM-1). The biological functions of these adhesion molecules are distinct. ICAM-1 binds leukocytes via their β2 integrins and can therefore serve to recruit both granulocytes and monocytes from the bloodstream to sites of tissue injury, whereas VCAM-1 binds monocytes and lymphocytes expressing the integrins α4β1 and α4β7 specifically supporting the recruitment of chronic inflammatory cells (10,11). In this paper we have investigated whether there is a direct connection between the p38 MAP kinase cascade and the expression of the endothelial adhesion molecules. By the use of the inhibitor SB203580 we demonstrate that the expression of VCAM-1, but not ICAM-1, is likely regulated by the p38 MAP kinase at the post-transcriptional level.

MATERIALS AND METHODS

Culture and FACS analysis of endothelial cells

Human umbilical vein endothelial cells (HUVEC) were cultured as previously described (12) in M199 (Flow Laboratories) supplemented with 10% newborn calf serum, 10% human serum kindly provided by the Red Cross Bloodbank, Rotterdam, The Netherlands, endothelial cell growth factor, 15 U/ml heparin, 50 U/ml penicillin, and 5 µg/ml streptomycin, under 95% air / 5 % CO_2. Confluent endothelial monolayers were treated as indicated in the legends, either in the presence or absence of SB203580, which was the generous gift of SmithKline Beecham, King of Prussia, USA. Cells were harvested and incubated for 30 min at 4°C with either monoclonal antibody clone IG11B1 (Monosan, Uden, The Netherlands) directed against VCAM-1, or clone MEM112 (Monosan) directed against ICAM-1, or control mouse IgG1κ . The second antibody was a fluorescein isothiocyanate conjugated rabbit anti-mouse Fab_2 fragment (Sigma Chemical Corp). Membrane antigen expression was analysed by fluorescence activated cell sorting (FACS) as previously described (13), and is expressed as linear fluorescence intensity (F.I.).

Polymerase chain reaction

Total RNA was isolated from 10^6 HUVEC stimulated with TNF (0 - 100 U/ml in complete M199) according to the method of Chomszynski *et al.* (14). cDNA was produced from 75 ng of each RNA extract by M-MLV reverse transcriptase (Promega) according instructions provided by

the manufacturer. Specific oligonucleotide primers (forward primer: 5'-CGGGATCCATCC-ACAAAGCTGCAAGAA-3', and reverse primer: 5'-GCGAATTCGCCACCACTCATCTC-GATTT-3'), yielding a polymerase chain reaction (PCR) product of 563 base pairs, were synthesized by Eurogentec according to the VCAM-1 cDNA sequence reported by Cybulsky *et al* (15). cDNA was amplified by 30 cycles with Goldstar DNA polymerase (Eurogentec) in a Perkin Elmer thermocycler 480 in a touch down protocol, in which the annealing temperature was gradually decreased from 72 °C to 53 °C. Homology of PCR product with the VCAM-1 cDNA was confirmed by sequence analysis.

Northern blot analysis

Total cellular RNA (20 µg) was size-fractioned using 1.2 % agarose formaldehyde gel in the presence of 1 µg ethidium bromide. The RNA was transferred onto Hybond and covalently linked by ultraviolet irradiation. Hybridizations were performed at 42 °C for 18 h in 50% deionized formamide, 1 M NaCl, 2.5 % dextran sulfate, 35 µg/ml denatured fish DNA in 20% P buffer (1% BSA, 1% polyvinylpyrrolidone, 1% Ficoll, 250 mM Tris-HCl, 0.5% Na-pyrophosphate, 5% SDS). The PCR product (approximately 50 ng cDNA) was radiolabeled with [α-^{32}P]dCTP (±50 µCi per hybridization)by the use of a labelling kit (Amersham). After hybridization, filters were washed with a final stringency of 2 x SSC (1 x = 150 mM NaCl, 15 mM Na citrate), 0.1% SDS at room temperature. Autoradiograms were quantified by phospho-imaging (Molecular Imaging System GS-363, Biorad).

MAPKAP kinase 2 activity assay

HUVEC were stimulated as indicated in the legends and washed three times with ice-cold phosphate buffered saline (PBS). Thereafter, cells were scraped, sedimented and stored at -80 °C prior to the assay. The pellet was re-dissolved in lysis buffer (0.27 M sucrose, 50 mM NaF, 20 mM Tris-acetate, 10 mM β-glycerophosphate, 5 mM pyrophosphate, 1 mM EGTA, 1 mM Na_3VO_4, 0.1 mM EDTA, 1 mM benzamidine, 0.2 mM phenylmethane-sulfonylfluoride, 2 µg/ml leupeptin, 1% Triton X-100 and 0.1% β-mercaptoethanol, pH=7.0) and incubated for 15 minutes at 0 °C. Lysates were cleared by centrifugation and the supernatants were incubated with an anti-MAPKAP kinase 2 antiserum over night prior to addition of protein A-Sepharose. One hour later the immuno-precipitates were washed three times with TBS (20 mM Tris-HCl, 0.154 M NaCl, pH= 7.5) containing 50 mM NaF, 1 mM Na_3VO_4 and 1% Triton X-100 and finally resuspended in a buffer consisting of 50 mM Na-β-glycerophosphate, 4 mM Mg-acetate, 1 mM EDTA, 20 µM H7 (Calbiochem), 20 µM HA1077 (Calbiochem), 2.5 µM protein kinase A inhibitor (Gibco), and 0.4 mg/ml Hsp25. The reaction was started by the addition of ATP (0.1 mM ATP containing 2 µCi γ-^{32}P-ATP) and terminated 10 minutes later by the addition of SDS-stopmix. Samples were subjected to SDS-PAGE and radioactivity in the Hsp25-band was quantitated by phospho-imaging as described above.

Hsp27 phosphorylation

HUVEC were washed twice with phosphate-free modified-Earles and incubated in the same medium containing 0.4 mCi/ml $^{32}PO_4^{3-}$ (Amersham) for 3 h. Thereafter, the cells were stimulated, washed 3 times (150 mM NaCl, 20 mM Na_3PO_4, pH=7.4, 0 °C) and Hsp27 phosphorylation was determined as described by Cuenda et al. (5). Briefly, after solubilization of the cells (lysis buffer, 10 minutes), the lysates were collected, cleared by centrifugation and protein G-Sepharose- conjugated anti-Hsp27 antibodies (StressGen, Victoria, Canada) were added to the supernatants. After 2 h of incubation at 0 °C, the beads were centrifuged, washed 3 times with 1 ml lysis buffer containing 0.5 M NaCl and twice with lysis buffer, resuspended in sample mix and subjected to SDS-PAGE. Radioactivity in the Hsp27 band was quantitated by phospho-imaging as described above.

RESULTS

Stimulation of the p38 MAP kinase cascade by TNF in endothelial cells

To study whether the p38 MAP kinase cascade is linked to the TNF stimulated expression of endothelial leukocyte adhesion molecules, we first analysed the process of activation of p38 MAP kinase by TNF in HUVEC. For that reason we stimulated these cells by TNF and measured the activity of the p38 MAP kinase target MAPKAP kinase-2 as well as the phosphorylation of the MAPKAP kinase-2 target, the small stress protein Hsp27, in these cells (Fig. 1).

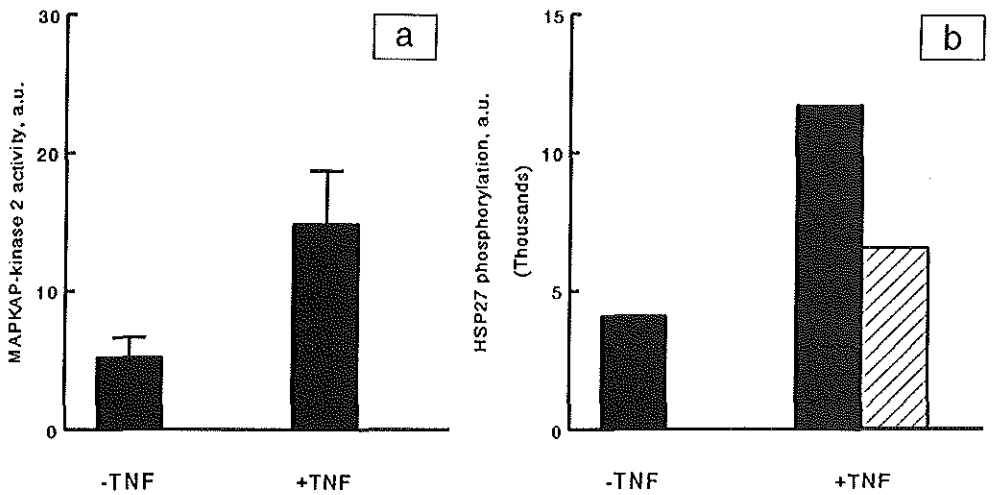

FIG 1. (A) Activation of MAPKAP kinase 2 activity in endothelial cells stimulated with 100 U/ml TNF. After 10 min of stimulation cells were lysed, and MAPKAP kinase 2 was immuno-precipitated. Enzymatic acitivity was assessed by Hsp25 phosphorylation *in vitro*. Data are presented as mean ± sem, n=3. (B) TNF stimulated Hsp27 phosphorylation in endothelial cells. $^{32}PO_4^{3-}$ pre-loaded cells were treated with or without 100 U/ml TNF for 10 min, in the absence dark bars) or presence (hatched bars) of 20 µM SB203580. Hsp27 was immuno-precipitated and subjected to SDS-PAGE. Radioactivity in the Hsp27 band was quantitated by phospo-imaging. The results are representative for two independent experiments.

As demonstrated in Fig. 1a TNF stimulates MAPKAP kinase-2 activity to about three fold in HUVEC. Furthermore, an increased phosphorylation of endogenous Hsp27 in these cells after TNF-treatment could be observed (Fig. 1b). The activation and phosphorylation of these downstream targets of p38 MAP kinase in response to TNF indicate a TNF-induced activation of p38 MAP kinase. To examine this further, we used the specific p38 MAP kinase inhibitor SB203580. After preincubation of the HUVEC with this inhibitor the TNF-induced

stimulation of MAPKAP kinase-2 activity (data not shown) and the phosphorylation of endogenous Hsp27 could be almost completely blocked (Fig. 1b). This finding confirms the notion that TNF activates the p38 MAP kinase cascade in HUVEC.

Role of p38 MAP kinase in the expression of endothelial cell adhesion molecules.

In parallel to the stimulation of the p38 MAP kinase cascade, TNF also stimulates the expression of the endothelial leukocyte adhesion molecules VCAM-1 and ICAM-1. At 6 h after stimulation of HUVEC with TNF an increased expression of both VCAM-1 and ICAM-1 can be detected by FACS analysis (Fig. 2).

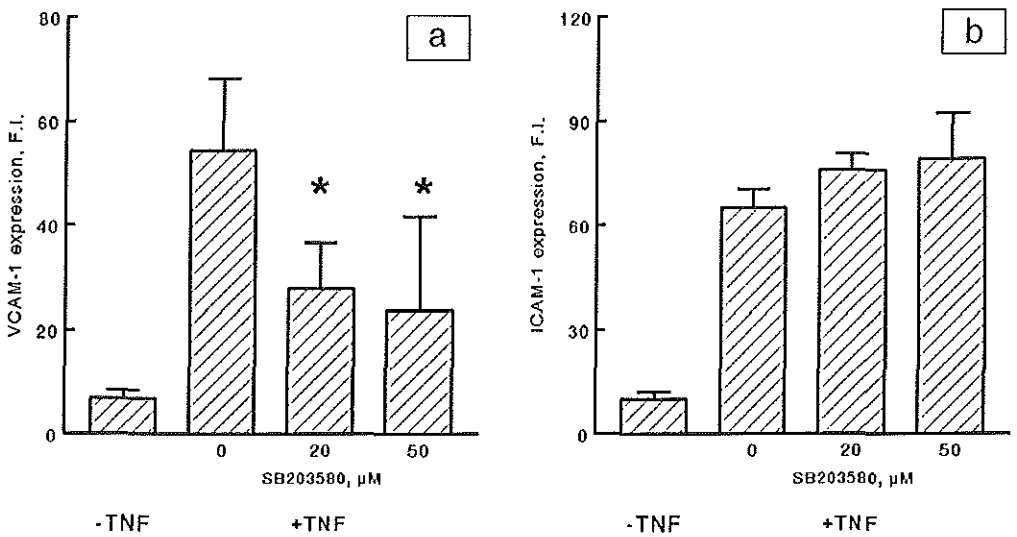

FIG 2. Effect of SB203580 on the TNF-induced expression of VCAM-1 (a) and ICAM-1 (b). Confluent monolayers of endothelial cells were pretreated with 20 or 50 µM SB203580 for 30 min, and subsequently stimulated with 100 U/ml TNF for 6 h also in the presence of SB203580. The expression of VCAM-1 and ICAM-1 was assessed by FACS analysis. The results are expressed as fluorescence intensities (mean ± sem, n=3-5). Asterisks indicates a significant difference from the untreated control. (Student's t-test, $p<0.05$).

To study the involvement of the p38 MAPK cascade in the regulation of VCAM-1 and ICAM-1 expression, we preincubated HUVEC with the p38 MAP kinase inhibitor SB203580. This inhibition of the p38 MAP kinase significantly reduced the expression of VCAM-1 at the surface of the cell (Fig. 2a). In contrast, ICAM-1 expression was not affected by inhibition of the p38 MAP kinase in endothelial cells (Fig. 2b).

Evidence for post-transcriptional regulation of VCAM-1 surface expression by the p38 MAP kinase cascade.

To understand the mechanism by which the p38 MAP kinase cascade regulates the VCAM-1 expression in endothelial cells we determined the VCAM-1 mRNA-level in TNF stimulated cells in the presence and absence of the p38 MAP kinase inhibitor SB203580. From the results presented in Fig. 3 it can be seen that SB203580 did not affect the accumulation of VCAM-1 mRNA, which gradually increased after 1 to 6 h of stimulation with TNF.

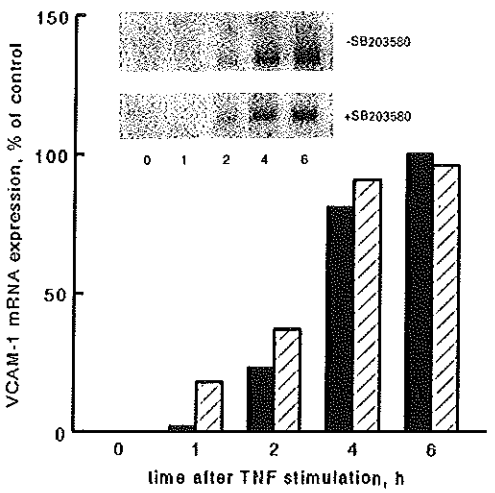

FIG 3. Time course of the TNF-induced expression of VCAM-1 mRNA. After 30 min pretreatment with 20 μM SB203580 or vehicle endothelial cells were stimulated with 100 U/ml TNF in the presence (hatched bars) or absence (dark bars) of SB203580. Total RNA was isolated and size-fractioned by denaturing 1.2 % agarose-formaldehyde gel electrophoresis, transferred to nitrocellulose and hybridized to ^{32}P-labeled human VCAM-1-specific cDNA (insert). Data are representative for three indepent experiments.

DISCUSSION

The recently identified p38 MAP kinase signalling cascade is activated under conditions of cellular stress, such as heat shock, hyperosmotic stimulation and after treatment with cytokines (1-3). In the present report we showed that the p38 MAP kinase pathway is functional in HUVEC, since (i) TNF stimulates the activity of MAPKAP kinase-2 and the subsequent phosphorylation of Hsp27 and (ii) TNF-induced stimulation of these downstream components of the p38 MAP kinase cascade is blocked by the p38 MAP kinase inhibitor SB203580. Analysis of the role for p38 MAP kinase in the expression of the endothelial cell adhesion molecules showed that the TNF-induced expression of VCAM-1 but not ICAM-1 is inhibited by the p38 MAP kinase inhibitor. An interesting feature of the regulatory mechanism involved in VCAM-1 surface expression was revealed by Northern blot analysis, since the TNF-induced accumulation of VCAM-1 mRNA clearly showed no difference in the presence or absence of SB203580. Hence, it can be assumed that the p38 MAP kinase pathway regulates VCAM-1 expression at the post-transcriptional level.

Previously, it has been shown that the p38 MAP kinase homologue CSBP regulates the biosynthesis of TNF by LPS-stimulated human monocytes at the translational level (4). It appears that the regulation of TNF translation is mediated through an AUUUA repeated motif in the 3'UTR of the TNF mRNA. Proteins binding to these AUUUA regions may be the target of the CSBP phosphorylation cascade (16,17). However, at present it is unclear whether p38 MAP kinase acts on the translation of VCAM-1 mRNA in a similar fashion, and whether or not MAPKAP kinase-2 and/or phosphorylated Hsp27 are involved in this process.

In the present study we have demonstrated the early activation of the p38 MAP kinase signalling cascade in endothelial cells stimulated with TNF. It seems plausible that the relatively late post-transcriptional regulation of VCAM-1 protein expression requires either a sustained activation of this signalling cascade (18), or the relatively stable phosphorylation of a substrate of the p38 MAP kinase pathway. In this respect it is relevant to indicate that TNF-action in fibroblasts has been shown to involve the inhibition of several protein phosphatases, which would increase the half-life of a putative phosphorylated intermediate (19). It is of note that the complete inhibition of the p38 MAP kinase could only partially decrease VCAM-1 protein expression. This indicates that p38 MAP kinase regulates only part of this post-transcriptional control and that other signalling cascades contribute as well.

The transcriptional regulation of VCAM-1 expression has been studied quite extensively and is likely to involve the assembly of a unique transcription activation complex consisting of the

inducible transcription factors NFκB, IRF-1 and constitutive transcription factor Sp-1 (20). However, in the present study we demonstrate for the first time that VCAM-1 protein expression by TNF-stimulated endothelial cells is also regulated at the post-transcriptional level. ICAM-1 expression on the other hand does not seem to depend on p38 MAP kinase activity.

ACKNOWLEDGEMENTS:

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REFERENCES

1. Marshall C.J. (1995) Specificity of receptor tyrosine kinase signalling: transient versus sustained extracellular signal-regulated-kinase action. *Cell* 80,179-185.
2. L'Allemain G. (1994) Deciphering the MAP kinase pathway. *Prog. Growth Factor Res.* 5,291-334.
3. Cano E., Mahadevan L.C. (1995) Parallel signal processing among mammalian MAPK's. *Trends Biochem. Sci.* 20:117-122.
4. Lee J.C., Laydon J.T., McDonnell P.C., Gallagher T.F., Kumar S., Green D., McNulty D., Blumenthal M.J., Heys J.R., Landvatter S.W., Strickler J.E., McLaughlin M.M., Siemens I.R., Fisher S.M., Livi G.P., White J.R., Adams J.L. and Young P.R. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 372, 739-746.
5. Cuenda A., Rouse J., Doza Y.N., Meier R., Cohen P., Gallagher T.F., Young P.R. and Lee J.C. (1995) SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* 364, 229-233.
6. Stokoe D., Engel K., Campbell D.G., Cohen P. and Gaestel M. (1992) Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins *FEBS Lett.* 313, 307-313.
7. Rouse J., Cohen P., Trigon S., Morange M., Alonso-Llamazares A., Zamanillo D., Hunt T., Nebreda A.R. (1994) *Cell* 78, 1027-1037.
8. Engel K., Ahlers A., Brach M.A., Herrmann F., Gaestel M. (1995) MAPKAP kinase 2 is activated by heat shock and TNF: in vivo phosphorylation of small heat protein results form the stimulation of the MAP kinase cascade. *J. Cell. Biochem.* 57, 321-330.
9. Arrigo A.P., Michel M.R. (1991) Decreased heat- and tumor necrosis factor-mediated hsp28 phosphorylation in the thermotolerant Hela cells. *FEBS Lett.* 282,152-156.
10. Springer T. (1995) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76, 301-314.
11. Elices M., Osborn L., Takada Y., Crouse C., Luhowskyj S., Hemler M., Lobb R. (1990) VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site dstinct from the VLA-4/fibronectin binding site. *Cell* 60, 577-584.
12. Pietersma A., de Jong N., Koster J.F. and Sluiter W. (1994) Effect of hypoxia on the adherene of granulocytes to endothelial cell *invitro. Am J Physiol (Heart Circ Physiol)* 39, H874-H879.
13. Pietersma A., Kofflard M., de Wit L.E.A., Stijnen T., Koster J.F., Serruys P.W., Sluiter W. (1995) Late lumen loss after coronary angioplasty is associated with the activation status of circulating phagocytes before treatment. *Circulation* 91, 1320-1325.
14. Chomczynski P., Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocynate-phenol chloroform extraction *Anal. Biochem.* 162, 156-159.
15. Cybulsky M., Fries J.W.U., Williams A.J., Sultan P., Eddy R., Byers M., Shows T., Gimbrone M.A., Collins T. (1991) Gene structure, chromosomal location, and basis for alternative mRNA splicing of the VCAM-1gene. *Proc. Natl. Acad. Sci. USA* 88, 7859-7863.

16. Young P.R., Mc Donnell P., Dunnington D., Hand A., Laydon J., Lee J.C. (1993) Bicyclic imidazoles inhibit IL-1 and TNF production at the protein level. Agents Action 39, C67-C69.
17. Lee J.C. and Young P.R. (1996) Role of CSBP/p38/RK stress response kinase in LPS and cytokine signalling mechanisms. *J. Leukoc. Biol.* 59, 152-157.
18. Meloche S., Pagès G., Pouysségur J. (1992) Functional expression and growth factor activation of an epitope-tagged p44 mitogen-activated protein kinase, p44mapk. *Mol. Biol. Cell* 3, 63-70.
19. Guy G.R., Cao X., Chua S.P., Tan Y.H. (1992) Okadaic acid mimics multiple changes in early protein phosphorylation and gene expression induced by tumor necrosis factor or interleukin –1. *J. Biol. Chem.* 267(3), 1846-1852.
20. Collins T., Read M.A., Neish A.S., Whitley M.Z., Thanos D., Maniatis T. (1995) Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine inducible enhancers.*FASEB J.* 9, 899-909

8 The role of iron in the expression of VCAM-1

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ABSTRACT

The accumulation of VCAM-1 mRNA in endothelial cells can specifically be inhibited by antioxidants, which indicates that reactive oxygen species (ROS) are necessary for the transcriptional activation of VCAM-1. Theoretically the low molecular weight (LMW) iron pool could control the metabolism of ROS inside the cell and thus VCAM-1 expression. However, a direct signalling role of the LMW iron pool is unlikely, since we demonstrate here that the LMW pool does not change in magnitude upon stimulation with TNF. In apparent contradiction, the iron chelator desferrioxamine inhibited the accumulation of VCAM-1 protein (but not that of ICAM-1). Suprisingly, this treatment had no effect on the accumulation of VCAM-1 mRNA nor on the nuclear translocation of the transcription factor NFκB. Studies on the incorporation of ^{35}S-methionine revealed that the translation of the VCAM-1 mRNA was inhibited to about 50%. Apparently, desferrioxamine inhibits an iron-dependent enzyme involved in the translational control of VCAM-1 expression. A likely candidate the cytoplasmic aconitase which functions as a switch in the control of the cellular iron homeostasis could be excluded. Although desferrioxamine inactivates this iron-sulfur cluster containing enzyme, no functional iron responsive elements could be detected in the 5' or 3' untranslated region of the of the VCAM-1 mRNA. The inhibitory effect of desferrioxamine on the expression of VCAM-1 is additive to that of the specific p38 MAP kinase inhibitor SB203580. This suggests that an iron-dependent enzyme is involved in a signalling cascade that diverts upstream of the p38 MAP kinase and controls the translation of VCAM-1.

INTRODUCTION

There is increasing evidence to suggest that a number of extracellular ligands require reactive oxygen species (ROS) to stimulate intracellular signalling, and to transmit their signal to the nucleus (1,2). For example, the ROS hydrogen peroxide has been implicated in the signal transduction leading to the activation of the ubiquitous transcription factor NFκB (3). The activation of NFκB plays an important role in the inflammatory response of endothelial cells. Central to this response is the accumulation of circulating leukocytes, which can be stimulated by inflammatory cytokines like TNF. In endothelial cells TNF induces the expression of the vascular cell adhesion molecule-1 (VCAM-1), and increases the expression of the intercellular cell adhesion molecule-1 (ICAM-1). The biological functions of these adhesion molecules differ. ICAM-1 binds leukocytes via their β2 integrins and can therefore recruit both granulocytes and monocytes from the circulation, whereas VCAM-1 binds monocytes and lymphocytes expressing the integrins α4β1 and α4β7 supporting the recruitment of chronic inflammatory cells (4,5). Although the activation of NFκB is an essential part of the stimulated expression of both ICAM-1 and VCAM-1, the transcriptional activation of only VCAM-1 is sensitive to antioxidants, implicating the requirement for ROS in this process (6,7).

The intracellular low molecular weight (LMW) iron pool, also known as the chelatable iron pool, may play an important role in the metabolism of ROS inside the cell. In the presence of the transition metal iron the ROS hydrogen peroxide can readily be converted into the highly reactive hydroxyl radical. The production of the hydroxyl radical is potentially hazardous to cells since it induces lipid peroxidation and the destruction of biological substrates (8). Therefore the production of this short-lived radical needs to be minimized. Although it seems plausible that the magnitude of the LMW iron pool can control/ regulate the ultimate fate of hydrogen peroxide in the cell by determining the rate of hydroxyl radical formation, this has not yet been proven.

In addition to a possible role of iron in regulating the fate of hydrogen peroxide in the cell there is ample proof for a regulatory role of the LMW iron pool in the intracellular iron metabolism (9). This regulatory mechanism involves the cytosolic iron responsive protein (IRP), which can sense iron present in the LMW pool (9). In the presence of iron this protein exhibits aconitase activity, whereas the aconitase activity is lost once the LMW iron pool becomes depleted (10). Under this latter condition IRP becomes an mRNA binding protein,

which coordinately represses the translation of the mRNA for ferritin and increases the stability of the mRNA for the transferrrin (Tf) receptor, leading to an increased uptake of iron in the cell (10).

These interesting aspects of the cell LMW iron pool have led us to study the putative role of iron in the TNF-stimulated expression of VCAM-1 by endothelial cells. To that end we have used the iron chelator desferrioxamine and established a role for iron in the post-transcriptional control of VCAM-1 protein expression. We have extended these studies in order to more precisely describe the role of iron, and have discriminated between the direct effect of this chelator on the LMW pool and the possibility that this compound chelates iron from the active site of one or more proteins.

MATERIALS AND METHODS

Culture and FACS analysis of endothelial cells

Human umbilical vein endothelial cells (HUVEC) were cultured as previously described (11) according to the method described by Jaffe *et al.* (12) in M199 (Flow Laboratories) supplemented with 10% newborn calf serum, 10% human serum kindly provided by the Red Cross Bloodbank, Rotterdam, The Netherlands, endothelial cell growth factor, 15 U/ml heparin, 50 U/ml penicillin, and 5 µg/ml streptomycin, under 95% air- 5 % CO_2. Confluent endothelial monolayers were treated as indicated in the legends. Cells were detached and incubated for 30 min at 4°C with either monoclonal antibody clone IG11B1 (Monosan, Uden, The Netherlands) directed against VCAM-1, or clone MEM112 (Monosan) directed against ICAM-1 as described before (10). The second antibody was a fluorescein isothiocyanate conjugated rabbit anti-mouse Fab_2 fragment STAR9 (Serotec, Oxford, UK). Membrane antigen expression was analysed by fluorescence activated cell sorting (FACS) as previously described (10), and is expressed as median linear fluorescence intensity (F.I.).

Northern blot analysis

Total cellular RNA (20 µg) was size-fractioned using 1.2 % agarose formaldehyde gel in the presence of 1 µg ethidium bromide. The RNA was transferred onto Hybond and covalently linked by ultraviolet irradiation. Hybridizations were performed at 42 °C for 18 h in 50% deionized formamide, 1 M NaCl, 2.5 % dextran sulfate, 35 µg/ml denatured fish DNA in 20% P buffer (1% BSA, 1% polyvinylpyrrolidone, 1% Ficoll, 250 mM Tris-HCl, 0.5% Na-pyrophosphate, 5% SDS). The VCAM-1 probe has been described in detail before (13). Approximately 50 ng of cDNA was radiolabeled with [α-^{32}P]dCTP (±50 µCi per hybridization) by the use of a labelling kit (Amersham). After hybridization, filters were washed with a final stringency of 0.2 x SSC (1 x = 150 mM NaCl, 15 mM Na citrate), 0.1% SDS at room temperature (rT). Autoradiograms were quantified by phospho-imaging (Molecular Imaging System GS-363, Biorad).

NFκB: Nuclear extracts were prepared according to the method of Schreiber et al (14) and next subjected to SDS-PAGE (10%) and Western blotting. The presence of NFκB was revealed by incubating the blots with 1:1000 rabbit anti-human NFκB p65 (Rel A) (Rockland, Gilbertsville) under rotation at rT overnight, followed by reaction with 1:3000 peroxidase-

conjugated anti-rabbit IgG (Santa Cruz Biotechnology) during 2 h and ECL Western blotting detection reagents (Amersham Life Science).

Determination of the low molecular weight iron pool

The low molecular weight iron pool in endothelial cells was assessed according to the method described in detail by Epsztejn *et al* (9). This method is based on the fluorescence of calcein (CA), which can bind intracellular iron present in de low molecular weight (LMW) pool resulting in the quenching of CA-fluorescence. The LMW iron pool of the endothelial cells equals the sum of the concentration of the CA-iron complex [CA-Fe] and the free iron concentration [Fe]. To quantitate the LMW iron pool the following parameters have to be determined experimentally, i.e., the K_d of CA-Fe, the total intracellular CA concentration $[CA]_t$, and [CA-Fe].

[CA-Fe] can be revealed by the use of the permeant chelator α,α'-bipyridyl (BIP), which removes iron from the CA-iron complex resulting in an increase in normalized CA fluorescence intensity (ΔF). The final concentration of BIP used was 150 μM. ΔF provides the means for calculating the cell [CA-Fe], which is given by $[CA\text{-}Fe]= \Delta F*[CA]_t$. The intracellular $[CA]_t$ is calculated from a calibration curve of free CA, the number of endothelial cells per mL, and their mean cell volume (amounting to 2853 ± 492 fL as determined with the CASEY® 1, Model TTC, Schärfe System GmbH, Reutlingen, Germany) and amounted to 32.5 μM. To determine the apparent K_d of the CA-Fe complex endothelial cells were harvested by trypsin treatment and resuspended in M199, with albumin (1 mg/ml) and without $NaHCO_3$. Next, the cells were loaded with 0.27 μM of CA-acetoxymethylester (AM) for 5 min at 37°C, permeabilized with ionophore (1 μM A23187), and titrated with Co(II). We used cobalt chloride instead of ferrous ammonium sulphate. This approach yields essentially similar results as with Fe(II) under anaerobic conditions (9), but is much more convenient. The apparent K_d was obtained by nonlinear regression analysis of the dependency of ΔF on the [Co(II)] given by the equation $\Delta F=K_d/(K_d+[Co(II)])$, and amounted to 0.21 ± 0.09 μM. Next, the $[Fe]_{free}$ can be calculated from the relation: $[Fe]=K_d*[CA\text{-}Fe]/([CA]_t -[CA\text{-}Fe])$.

Aconitase activity

Endothelial cells (150 cm^2) were trypsinized, pooled and washed in phosphate buffered saline containing 1.0 mM Ca^{2+} and 0.5 mM Mg^{2+} (PBS^{++}), sedimented (5 min at 400g) and kept at

4°C during the entire procedure. Cells were resuspended in 30 mM triethanolamine (TEA), 0.15 M NaCl, pH 7.2. Cells were permeabilized by the consecutive addition of 0.007% digitonin and 0.2 % Triton X-100. The supernatant which was acquired after centrifugation for 15 min at 4300g was assayed for aconitase activity. To that end 0.5 ml of supernatant was incubated in the presence 200 nmoles of cis-aconitate in TEA buffer with 0.01% bovine serum albumin at rT. The decrease in absorbance at 240 nm in time was used as a measure of aconitase activity.

^{35}S-methionine incorporation

Endothelial cells in 6 wells plates were pretreated with or without 1.0 mM desferrioxamine for 1 h in complete culture medium M199 and next stimulated with 100 U/ml TNF for 1 h. After washing with PBS, methionine-free MEM (GIBCO) of which the amino acid composition had been adjusted to that of MEM was added to each well and 40 µCi of Trans ^{35}S-label (ICN Biomed Inc, Irvine, CA). Then the cells were further stimulated with 100 U/ml TNF in the presence or absence of 1.0 mM desferrioxamine for 4 h. Immediately thereafter the cells were washed with PBS and harvested in Puck's A (GIBCO) with 0.2 % EDTA, 0.05% sodium azide, and 10% FCS. Cells were then washed with PBS with 2 mM PMSF and lysed in lysisbuffer containing 0.5% Nonidet P40, 4 mM EDTA, 50 mM Tris, 150 mM NaCl, 1 mM PMSF for 18 h at 4°C, and stored at -20°C until further analysis.

Immunoprecipitation and SDS-PAGE

VCAM-1: The endothelial cell homogenate was subjected to centrifugation at 12000g for 15 min and the pellet discarded. The lysates were then incubated with (1:10) mAb IG11B1 directed against VCAM-1 for 18 h in lysis buffer at 4°C. Next, the lysates were incubated with 1:40 protein G sepharose for 1.5 h at 4°C under rotation. Antibody conjugated beads were pelleted and washed three times in PBS containing 0.1% nonidet, and placed in sample buffer with 5% mercaptoethanol. Samples were boiled for 5 min and analyzed by 10% sodium dodecylsulfate polyacrylamide gelelectrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene difluoride membrane (Boehringer Mannheim, Germany). Autoradiography of the incorporated ^{35}S-methionine was quantitated with the Molecular Imaging System GS-363 (Biorad). In order to study the effect of TNF on the LMW iron pool tracings of CA fluorescence were obtained. Endothelial cells in 24-wells plates were loaded with CA-AM (0.5 µM) in culture medium for 30 min at 37°C, and then washed two times

with HBSS. The fluorescence was read with time in the presence or absence of TNF using a microplate fluorimeter (Cytofluor® series 4000, B&L Systems, Maarssen, The Netherlands) with excitation and emission wavelengths set at 485 nm and 530 nm, respectively.

Assay of hydrogen peroxide production by endothelial cells

The production of hydrogen peroxide by endothelial cells was assessed as described previously (15). In short, endothelial cells in 25 cm^2 wells culture plates were incubated in Hanks Balanced Salt Solution (HBSS), pH 7.4, with 0.1 mM homovanillic acid and 1 U/ml horse radish peroxidase (Boehringer Mannheim, Germany) for 0 to 120 min at 37°C in the presence or absence of 100 U/ml TNF. The reaction was stopped by the addition of 0.1 M glycine/25 mM EDTA buffer, pH 12. The fluorescence of the supernatants was read in a fluorimeter (Perkin Elmer Luminescence Spectrometer LS-3B) with excitation and emission wavelengths set at 312 nm and 420 nm, respectively.

Fluorimetric analysis of lipid peroxidation

To measure lipid peroxidation in vital endothelial cells the naturally fluorescent cis-parinaric acid (Molecular Probes, Eugene, OR) was used. Endothelial cells in 75 cm^2 culture flasks were washed with PBS^{++}, and incubated with 5 µM cis-parinaric acid for 1 h at 37°C, essentially according to the procedure described by Hedley *et al.* (16). Endothelial cells were detached, washed with PBS^{++}, pelleted and resuspended in PBS^{++} at 1 x 10^6 cells/ ml. After stimulating the cells with 100 U/ ml TNF the fluorescence of parinaric acid was monitored continuously for 90 min in a fluorimeter (Perkin Elmer) at 37°C. Excitation and emission wavelengths were set at 325 nm and 405 nm, respectively. As a positive control the effect of 1.0 mM cumene hydroperoxide (Sigma) on the fluorescence of the endothelial cells was assessed.

Statistical analysis

Data are expressed as the means ± standard error of mean (SE). The differences between means were assessed by Student's *t*-test. Significance was determined at the 95% confidence level.

RESULTS

Effect of desferrioxamine on the expression of VCAM-1 by endothelial cells

To study the role of iron in the expression of VCAM-1, after a pre-incubation period of 30 min with increasing concentrations of the iron chelator desferrioxamine, endothelial cells were stimulated for 18 h with 100 U/ml of TNF in the presence of desferrioxamine.The results presented in Fig. 1show that desferrioxamine inhibited the TNF-stimulated expression of VCAM-1 in a dose dependent manner. At higher concentrations of desferrioxamine (up to 0.6 mM) the expression of VCAM-1 was maximally reduced to about 30% of the control. The inhibitory effect of desferrioxamine could be completely prevented by saturating desferrioxamine with iron beforehand (Fig. 1). The inhibitory effect of desferrioxamine on the TNF-induced VCAM-1 expression was already significant after 6 h of stimulation (see below).

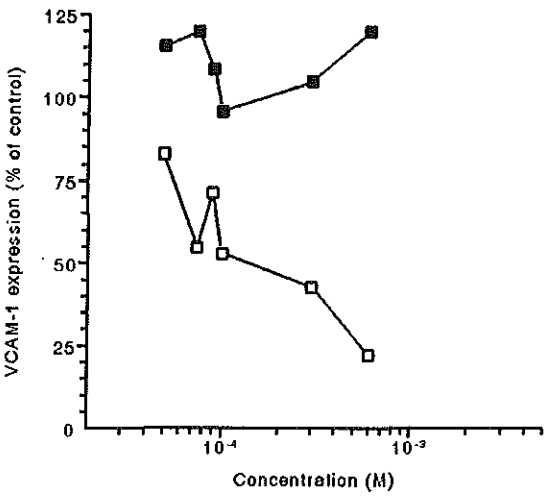

Fig 1. Effect of the iron chelator desferrioxamine (open squares) on the expression of VCAM-1. Endothelial cells were preincubated with desferrioxamine 30 min, next stimulated with TNF (100 U/ml) in the presence of the chelator, and the expression of VCAM-1 was assessed. Data are representative of three independent experiments. The inhibition of VCAM-1 expression by desferrioxamine was prevented by saturating desferrioxamine with iron (filled squares).

To assess whether the effect of desferrioxamine was specific for VCAM-1 we also studied the expression of ICAM-1 in the presence of desferrioxamine. The results in Fig. 2 show that increasing concentrations of desferrioxamine up to 1 mM did not significantly decrease ICAM-1 expression in endothelial cells.

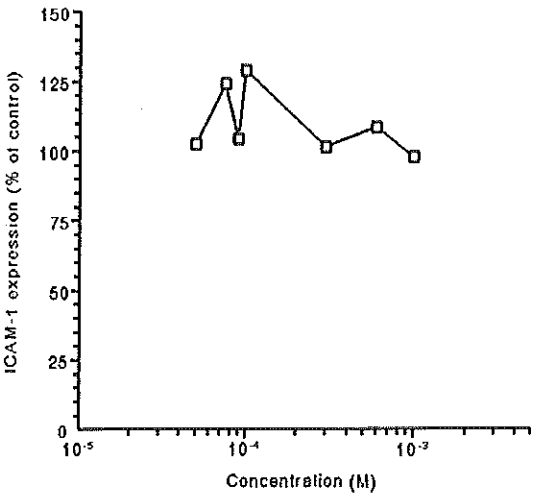

Fig 2. Effect of desferrioxamine on the expression of ICAM-1. Endothelial cells were preincubated with desferrioxamine for 30 min, next stimulated with TNF (100 U/ml) in the presence of desferrioxamine, and the expression of ICAM-1 was assessed. Data are representative of three independent experiments.

Effect of desferrioxamine on the transcriptional regulation of VCAM-1 expression

To study at which level iron is involved in the TNF stimulated induction of VCAM-1 expression we set out to determine the effect of desferrioxamine on the accumulation of VCAM-1 mRNA after 0 to 6 h of stimulation with TNF. Equal amounts of RNA were subjected to Northern blot analysis and the integrity of the RNA samples was confirmed by ethidium bromide staining of 28S rRNA in the gel (data not shown).

The results presented in Fig. 3 demonstrate that desferrioxamine did not affect the accumulation of VCAM-1 mRNA in endothelial cells. Transcription of the VCAM-1 gene is strictly dependent on the translocation of the transcription factor NFκB.

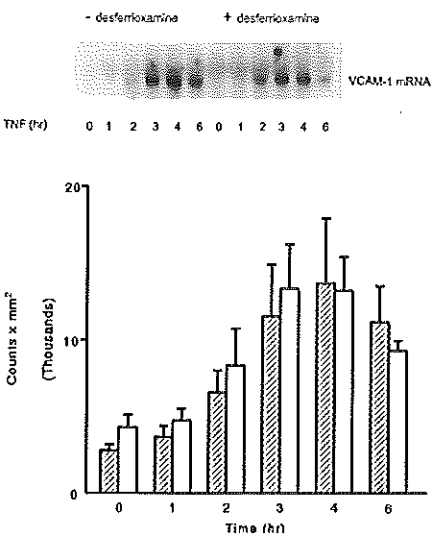

Fig 3. Effect of desferrioxamine on the TNF-induced expression of VCAM-1 mRNA. Endothelial cells were preincubated with desferrioxamine (0 - 600 μM), and next stimulated with 100 U/ml TNF in the presence (open bars) or absence (hatched bars) of desferrioxamine. The accumulation of VCAM-1 mRNA was assessed by Northern blot analysis. The results represent the mean and SE of three independent experiments. Inserted photograph represents a typical experiment.

The results presented in Fig. 4 show that stimulation with TNF stimulates the nuclear translocation of the NFκB family member p65 in endothelial cells, and that this translocation of p65 is not decreased by treating the endothelial cells with desferrioxamine for increasing periods of time.

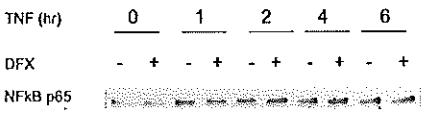

Fig 4. Effect of desferrioxamine on the nuclear translocation of NFκB. Endothelial cells were stimulated with 100 U/ml TNF in the presence or absence of 1 mM desferrioxamine for increasing periods of time. Nuclei were isolated and analysed for the presence of the p65 DNA binding subunit by Western blotting.

Effect of desferrioxamine on the translation of VCAM-1 mRNA

To determine whether desferrioxamine decreased the translation of VCAM-1 mRNA into VCAM-1 protein, endothelial cells were stimulated for 5 h with TNF in the presence of ^{35}S-methionine. Next, the incorporation of ^{35}S-methionine in the immunoprecipitated VCAM-1 protein was assessed by autofluorography. The results presented in Table 1 show that desferrioxamine significantly decreased the incorporation of ^{35}S-methionine in VCAM-1 to about 50%. This effect was similar to the effect of desferrioxamine on the surface expression of VCAM-1, which was significantly reduced to 50% of the control under the applied conditions (Table 1).

Table 1. Effect of desferrioxamine on the endothelial vascular cell adhesion molecule-1 protein expression.

Analysis	- desferrioxamine	+ desferrioxamine	P-value
FACS	332 (36)	170 (15)	0.036
^{35}S-methionine-incorporation	12500 (4600)	6800 (2500)	0.046

Endothelial cells were pretreated with or without 1.0 mM desferrioxamine for 1 h, and next stimulated with TNF in the presence of ^{35}S-methionine and 0 - 1.0 mM desferrioxamine. Next, the cells were either harvested and analysed by FACS-analysis after 6 h of TNF stimulation, or lysed and immunoprecipitated after 5 h of TNF stimulation as described. The incorporation of ^{35}S-methionine into the immunoprecipitated VCAM-1 was assessed after SDS-PAGE and Western blotting of the samples. Statistical significance of four separate experiments was assessed by Student's *t*-test for paired data.

Effect of desferrioxamine and TNF on the endothelial LMW iron pool

To determine the effect of desferrioxamine on the LMW iron pool endothelial cells were incubated with 1 mM desferrioxamine for increasing periods of time (0, 30, 60 and 300 min). We found that under normal conditions the fraction of the LMW iron pool that bound to CA amounted to 2.31 ± 0.88 μM of iron, and that only less than 0.03 μM of iron remained free. After 30 min of incubation desferrioxamine chelated about 60% of the amount of iron present in the LMW pool, and after 60 min no LMW iron could be detected.

If the LMW pool of endothelial cells has a function in the intracellular signalling stimulated by TNF, the fluorescence of CA is expected to change. The results presented in Fig. 5 show that this was not the case indicating that TNF does not mobilize cellular iron into the LMW pool.

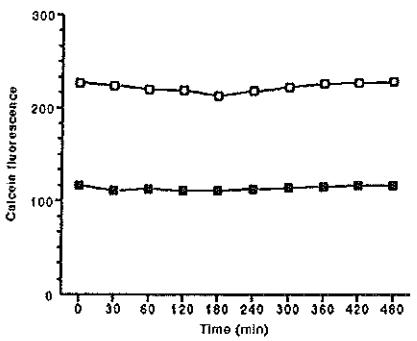

Fig 5. Effect of TNF on the low molecular weight iron pool in endothelial cells. Endothelial cells were loaded with calcein acetomethylester. The cells were washed with HBSS and treated with 100 U/ml TNF in HBSS. The calcein fluorescence was read with excitation and emission wavelengths set at 485 nm and 530 nm, respectively. Results of two independent experiments.

Effect of desferrioxamine on the endothelial aconitase activity

To investigate whether desferrioxamine decreases endothelial aconitase activity, cells were incubated with desferrioxamine for 30 min, and next the aconitase activity was assessed in the presence of the iron chelator. The results presented in Fig. 6 show that desferrioxamine significantly inhibited the aconitase activity of endothelial cells to about 50% of the control.

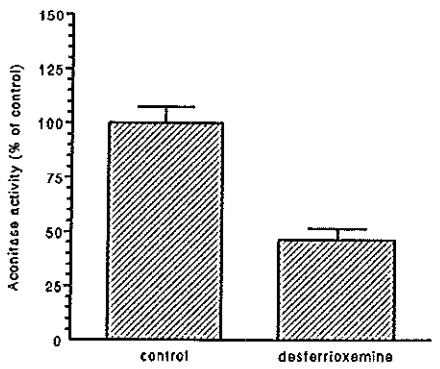

Fig 6. Effect of the iron chelator desferrioxamine on the endothelial aconitase activity. Endothelial cells were preincubated with desferrioxamine (600 μM), next cells were detached and aconitase activity was assessed as the consumption of cis-aconitate in permeabilized cells.

Does the 5'-untranslated region of VCAM-1 mRNA contain iron responsive elements?

To investigate whether the translational regulation of VCAM-1 involves the activity of the iron responsive regulatory protein IRP we searched the entire VCAM-1 mRNA sequence derived from the cDNA originally reported by Cybulsky *et al.* (18) for classical and alternative iron responsive elements (IREs) (17). The results showed that the classical IRE 5'-CAGUG-3' sequence is present in exon V, located at nucleotide 2081 of the original cDNA sequence (18). And that the alternative sequence 5'-UAGUA-3' is present in the 3'UTR located at nucleotides 4689 and 4816 of the original sequence (18). However, none of the three regions identified here allowed the formation of a stem-loop structure with an unpaired C positioned 5' of the loop.

Effect of TNF on the hydrogen peroxide production by endothelial cells

To investigate the involvement of the ROS hydrogen peroxide as a second messenger in the intracellular signalling of VCAM-1 protein expression, we stimulated endothelial cells with TNF and assessed the extracellular production of hydrogen peroxide within 2 h after stimulation. The basal production of hydrogen peroxide amounted to 57 ± 11 pmol.min^{-1}.mg^{-1} cell protein. From the results presented in Fig. 7 it can be seen that this basal rate of hydrogen peroxide production did not increase upon stimulation with TNF.

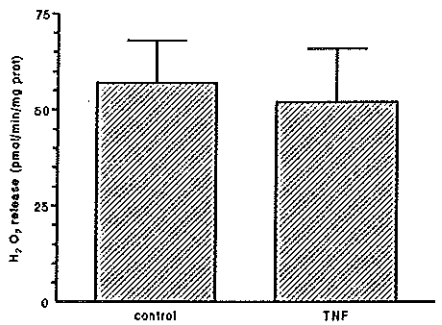

Fig 7. Effect of TNF on the extracellular release of hydrogen peroxide by endothelial cells. Cells were incubated with 0.1 mM homovanillic acid, and 1 U/ml horse radish peroxidase in the presence or absence of 100 U/ml TNF for 0 to 2 h at 37°C. The results are expressed as picomoles hydrogen peroxide/min/mg cell protein (mean ± SE, n=3).

Effect of TNF on the lipid peroxidation in the endothelial cells

To determine whether TNF stimulates the induction of VCAM-1 through an increase in lipid peroxidation, we studied the effect of TNF on the extent of lipid peroxidation in vital endothelial cells loaded with the fluorescent probe cis-parinaric acid. The results presented in Fig. 8 show that stimulation of the endothelial cells did not increase the oxidation rate of cis-parinaric acid, whereas the oxidation rate of the probe was increased when cells were incubated with the lipophilic cumene hydroperoxide.

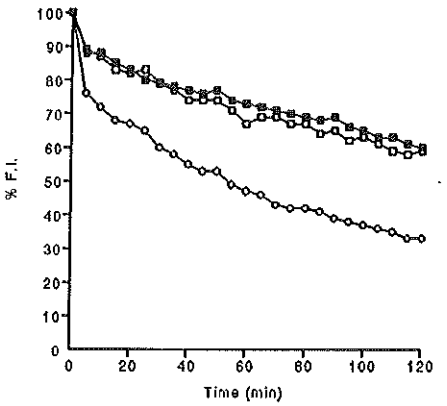

Fig 8. Effect of TNF on the lipid peroxidation in vital endothelial cells. Cells were incubated with 5 µM cis-parinaric acid for 1 h at 37°C, and the fluorescence of cis-parinaric acid was read for up to 2 h in the presence (filled squares) or absence (open squares) of 100 U/ml TNF, or in the presence of 1 mM cumene hydroperoxide (open circles). Excitation and emission wavelengths were set at 325 nm and 405 nm, respectively. Results are representative of two independent experiments

DISCUSSION

In the present study we have demonstrated for the first time the requirement for iron in the post-transcriptional control of VCAM-1 protein expression. We show here that the TNF-stimulated membrane expression of VCAM-1 on endothelial cells is decreased in the presence of the iron chelator desferrioxamine, without having an effect on the accumulation of VCAM-1 mRNA, and on the translocation of the requisite transcription factor NFκB. The effect of desferrioxamine could be completely abolished by saturating the chelator with iron beforehand. Since in the presence of desferrioxamine the ^{35}S-methionine incorporation into VCAM-1 protein is decreased to the same extent as the membrane expression of VCAM-1, we conclude that iron is required during the translational control of VCAM-1 protein expression.

The question arose whether the translational control of VCAM-1 requires iron present in the LMW pool or in a protein bound form. The results of our study indicate that the former possibility seems unlikely. In the first place TNF stimulation did not increase the magnitude of the LMW iron pool in endothelial cells. And secondly, TNF did not stimulate the endothelial production of hydrogen peroxide. Which is in accordance with data presented by Royall *et al* (19), who showed that TNF does not increase the intracellular concentration of hydrogen peroxide, nor its release by endothelial cells. Theoretically, an increase in either the LMW iron pool or the intracellular concentration of hydrogen peroxide could have stimulated lipid peroxidation. However, our results with the fluorescent probe cis-parinaric acid show that TNF does not increase lipid peroxidation in endothelial cells. We therefore conclude that it is unlikely that the LMW iron pool exerts a regulatory or signalling role in the expression of VCAM-1. Can protein-bound iron regulate the translation of VCAM-1 mRNA? We found that the activity of the 4Fe-4S cluster containing aconitase in endothelial cells was reduced to about 50% after 30 min of incubation with desferrioxamine. It has been well established that the intracellular level of iron is regulated through the iron responsive protein IRP, which exhibits cytoplasmic aconitase activity (9,10). When the intracellular iron levels are depleted IRP looses its aconitase activity, and becomes a mRNA binding protein interacting with typical stem-loop structures called iron responsive elements (IRE's) (17). Apo-IRP inhibits the translation of the ferritin H- and L-chain mRNA's through binding IRE's present in the 5'-untranslated regions of these messengers. At the same time apo-IRP promotes transferrin receptor synthesis through binding the 3' end of the transferrin receptor mRNA and enhancing its stability (10). This ultimately leads to the increased cellular uptake of iron. Endothelial

aconitase activity can in part be attributed to IRP. Could it be that the IRP also inhibits the translation of the VCAM-1 messenger? Although desferrioxamine was found to inhibit endothelial aconitase activity, the VCAM-1 mRNA does not contain any typical IRE's either in the 5' or 3' UTR. Therefore a role for IRP in the translational control of VCAM-1 can be excluded.

To ultimately identify the relevant iron containing protein involved in the translational regulation of VCAM-1 the specificity of the effect of desferrioxamine may provide us with a clue. In contrast to VCAM-1, ICAM-1 expression could not be inhibited by desferrioxamine. This suggests the existence of a mechanism which specifically regulates the translation of VCAM-1 mRNA. In that respect the translational control of the TNF biosynthesis is of interest. The regulation of TNF translation is mediated through an AUUUA repeated motif located in the 3'UTR of its mRNA (20,21). If in the appropriate nucleotide context these AUUUA regions not only determine mRNA stability, but also translational efficiency (22,23). Proteins that specifically bind to the site may repress translation initiation by interacting with the cap of the 5'-UTR of the mRNA (23,24). Lee and Young postulated that phosphorylation of this protein by the p38 MAP kinase cascade would relieve the translational repression of TNF (25). We have previously found that the p38 MAP kinase cascade regulates VCAM-1 but not ICAM-1 protein expression at the post-transcriptional level (13). Since AUUUA pentamers are present in the 3' UTR of VCAM-1 mRNA, this may suggest that the p38 MAP kinase phosphorylation cascade also is involved in the translational control of VCAM-1. However, complete inhibition of the p38 MAP kinase only partially decreased VCAM-1 protein expression indicating that p38 MAP kinase regulates only part of this translational control (13). In the present study we found that the inhibitory effects of SB203570 and desferrioxamine on the TNF-stimulated VCAM-1 expression are additive (results not shown). Apparently, the relevant enzyme that contains iron in its catalytic site and remains to be established is part of a separate signalling cascade.

At present we have shown for the first time the requirement for iron in the translational control of the TNF-stimulated VCAM-1 expression. This newly identified mechanism of translational control does not seem to be involved in the expression of ICAM-1.

ACKNOWLEDGEMENTS

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REFERENCES

1. Sundaresan, M., Z. Yu, V.J. Ferrans, K. Irani, and T. Finkel. 1995. Requirement for generation of hydrogen peroxide for platelet-derived growth factor signal transduction. *Science* 270:297.
2. Feng, L., Y. Xia, G.E. Garcia, D. Hwang, and C.B. Wilson. 1995. Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-α, and lipopolysaccharide. *J. Clin. Invest.* 95:1669.
3. Schreck, R., P. Rieber , and P.A. Baeuerle. 1991. Reactive oxygen intermediates as apparently widely used messenger in the activation of the NFκB transcription factor and HIV-1.*EMBO J.* 10:2247.
4. Springer, T. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76:301.
5. Elices, M., L. Osborn, Y. Takada, C. Crouse, S. Luhowskyj, M. Hemler, and R. Lobb. 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site disinct from the VLA-4/fibronectin binding site. *Cell* 60:577.
6. Marui, N., M.K. Offerman, R. Swerlick, C. Kunsch, C.A. Rosen, M. Ahmad, R.W. Alexander, and R.M. Medford. 1993. Vascular cell adhesion molecule-1 gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J. Clin. Invest.* 92:1866.
7. Weber, C., W. Erl, A. Pietsch, M. Ströbel, H.W.L. Ziegler-Heitbrock, and P.C. Weber. 1994. Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-κB mobilization and induction of vascular cell adheson molecule-1 in endothelial cells stimulated to generate radicals. *Arterioscler. Thromb.* 14:1665.
8. Halliwell, B. 1991. *Free Radicals and Food Additives.* Taylor & Francis, London, UK, p. 37.
9. Epsztejn, S., O. Kaklon, H. Glickstein, W. Breuer, and Z.I. Cabantchik. 1997. Fluorescence analysis of the labile iron pool of mammalian cells. *Anal. Biochem.* 248:31.
10. Hirling, H., B.R. Henderson, and L.C. Kühn. 1994. Mutational analysis of the [4Fe-4S] cluster converting iron regulatory factor from its RNA-binding form to cytoplasmic aconitase. *EMBO J.* 13:453.
11. Pietersma, A., N. de Jong, J.F. Koster, and W. Sluiter. 1994. Effect of hypoxia on the adherence of granulocytes to endothelial cells *in vitro. Am. J. Physiol. (Heart Circ Physiol).* 39:H874.
12. Jaffe, E.A., R.L. Nachman, C.G. Becker, and C.R. Minick. 1973. Culture of human endothelial cells derived from human umbilical veins: identification by morphologic and immunologic criteria. *J. Clin. Invest.* 52:2745.
13. Pietersma, A., B.C. Tilly, M. Gaestel, N. de Jong, J.C. Lee, J.F. Koster, and W. Sluiter. 1997. P38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. *Biochem. Biophys. Res. Commun.* 230:44.
14. Schreiber, E., P. Matthias, M.M. Müller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* 17: 6419.

15. Ruch, W., P.H. Cooper, and M. Baggiolini. 1983. Assay of hydrogen peroxide with homovanillic acid and horse radish peroxidase. *J. Immunol. Meth.* 63:347.
16. Hedley, D., and S. Chow. 1992. Flow cytometric measurement of lipid peroxidation in vital cells using parinaric acid. *Cytometry* 13:686.
17. Henderson, B.R., E. Menotti, C. Bonnard, and L.C. Kühn. 1994. Optimal sequence and structure of iron-responsive elements. *J. Biol. Chem.* 269:17481.
18. Cybulski, M.I., J.W.U. Fries, A.J. Williams, P. Sultan, R. Eddy, M. Byers, T. Shows, M. A. Gimbrone, and T.A. Collins. 1991. Gene structure, chromosomal location, and basis for alternative mRNA splicing of the VCAM1 gene. *Proc. Natl. Acad. Sci. USA.* 88:7859.
19. Royall, J.A., P.D. Gwin, D.A. Parks, and B.A. Freeman. 1992. Responses of vascular endothelial oxidant metabolism to lipopolysaccharide and tumor necrosis factor-κ. *Archiv. Biochem. Biophys.* 294:686.
20. Han, J., and B. Beutler. 1990. The essential role of the UA-rich sequence in endotoxin-induced cachectin/TNF synthesis. *Eur. Cytokine Net.* 1:71.
21. Han, J., G. Huez, and B. Beutler. 1991. Interactive effects of the tumor necrosis factor promotor and 3'-untranslated regions. *J. Immunol.* 146:1843.
22. Zubiaga, A.M., J.G. Belasco, and M.E. Greenberg. 1995. The nanomer is the key AU-rich sequence motif that mediates mRNA degradation. *Mol. Cell. Biol.* 15:2219.
23. Kruys, V., O. Marinx, G. Shaw, J. Deschamps, and G. Huez. 1989. Translational blockade imposed by cytokine-derived UA-rich sequences. *Science* 245:852.
24. Standart, N., and R.J. Jackson. 1994. Regulation of translation by specific protein/mRNA interactions. *Biochimie* 76:867.
25. Lee, J.C., and P.R. Young. 1996. Role of CSBP/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. *J. Leukoc. Biol.* 59:152.

9 Restenosis a special case of atherosclerosis progression

9.1 Introduction

In a progressive stage atherosclerotic plaques (stenosis) can obstruct coronary arteries and compromise the supply of blood. To alleviate the symptoms of obstructive stenosis different revascularization procedures have been developed. Percutaneous transluminal angioplasty or balloon dilation of a stenosed vessel was first performed by Gruentzig in the late 1970s (1). Two decades later worldwide, nearly one million patients receive PTA of a coronary artery each year (2). In the Netherlands alone the total number of PTA procedures has increased from about 7000 in 1989 to about 14000 in 1996 (3). Unfortunately, in 20 – 40 % of these cases the initial gain in luminal diameter is lost, due to the process of restenosis (4,5).

Characteristically restenosis develops in several different phases (2,6,7) over a period of three to seven months (8). Directly after PTA platelet deposition occurs. Due to the removal of endothelium platelets adhere to the exposed lipids and collagen matrix, aggregate and form thrombus. Platelet deposition subsides after 24 hours. The next phase is characterized by an invasion of granulocytes, monocyte/ macrophages and T-lymphocytes into the lesion. These chronic inflammatory cells are an important source of cytokines and growth factors, which stimulate smooth muscle cell migration in the subsequent phase of the healing response (4). Normally, vascular smooth muscle cells are in a quiescent contractile state. After stimulation they convert to a mobile synthetic phenotype and migrate toward the injury site, a process that is facilitated by degradatory enzymes like the metalloproteinases (6). Once in the damaged area the smooth muscle cells proliferate and secrete extracellular matrix proteins, which may cause intimal thickening at the PTA site. In the final phase of the healing response smooth muscle cells no longer proliferate, but secrete and organize the extracellular matrix (6).

Restenotic lesions generally have an a-cellular appearance and are composed for over 50% of extracellular matrix proteins (6). Also the proliferation rate of smooth muscle cells is rather low (9). Based on these and other data it has recently been proposed that the organization of the extracellular matrix, or remodeling, is an important determinant of the outcome of PTA (2,6). Remodeling is either unfavorable or favorable, and respectively results in vessel constriction or compensation of intimal formation in such a way that the lumen is preserved (6). Restenosis by definition is a loss in lumen diameter, and can be quantitated by the assessment of lumen renarrowing or lumen loss. However, when assessed angiographically

lumen renarrowing does not give detailed information on the composition of the restenotic lesion.

While smooth muscle cell proliferation, and synthesis and organization of the extracellular matrix may be important determinants of restenosis, early effects of the PTA treatment have a crucial contribution as well. Although restenosis occurs long after platelet aggregation and thrombus formation have waned, inhibition of platelet function gives a significant reduction in clinical events (10). The initial thrombus may provide a scaffolding that enhances the migration of smooth muscle cells, as well as providing a stimulus for smooth muscle cell proliferation (6). Also a prolonged activation of the inflammatory reponse may translate early responses into the relatively late clinical effects. Monocyte/ macrophage cells are probably present during the entire restenosis process (2). These cells are an important source of cytokines and growth factors, i.e., IL1, TNF, platelet derived growth factor (PDGF), and basic fibroblast growth factor (bFGF). Libby et al have proposed that PTA first activates cytokine gene expression by macrophages and/or smooth muscle cells within the plaque. This acute cytokine expression then elicits secondary, self-sustaining, and continuing autocrine and paracrine cytokine and growth factor expression by cells recruited into the lesion. In that manner the initial response can be amplified, which may explain the lag between injury and restenosis (4).

The amount of cytokine a monocyte can secrete upon stimulation is genetically determined (11,12). Still the cytokine production of circulating monocytes can vary in time due to the in vivo state of the immune response, and will be higher when the monocyte is in a primed state (13). This implies that the activation status of circulating monocytes can in part determine the degree of luminal renarrowing.This hypothesis is investigated in chapter 9.2 of this thesis.

References

1. Gruentzig A. Transluminal dilatation of coronary artery stenosis. Lancet 1978;i,263-266
2. Dalstra JAA, Reitsma JB. Hart-en vaatziekten in Nederland. Cijfers over ziekte en sterfte. P17-p23 Den Haag 1997.
3. Libby P, Schwartz D, Brogi E, Tanaka H, Clinton SK. A cascade model for restenosis. A special case of atherosclerosis progression. Circulation 1992;86[suppl III]:III-47-III-52
4. Califf RM, Fortin DF, Frid DJ, Harlan WR, Ohman EM, Bengston JR, Nelson CL, Tcheng JE, Mark DB, Stack RS. Restenosis after coronary angioplasty: an overview. J Am Coll Cardiol. 1991;17:2B-13B
5. Libby P, Tanaka H. The molecular bases of restenosis. Progress in Cardiovascular Dis. 1997;40,97-106
6. Faxon DP, Coats W, Currier J. Remodeling of the coronary artery after vascular injury. Progress in Cardiovascular Dis. 1997;40,129-140
7. Schwartz RS, Holmes DR, Topol EJ. The restenosis paradigm revisited: an alternative proposal for cellualr mechanisms. J Am Coll Cardiol. 1992;20:1284-1293
8. Serruys PW, Luijten HE, Beatt KJ et al Incidence of restenosis after successful coronary angioplasty: a time related phenomenon. Circulation 1988;77:361-71.
9. O'Brien ER, Alpens CE, Stewart DK, et al. Proliferation in primary and restenotic coronary atherectomy tissue. Implications for anti-proliferative therapy. Cir Res 1993;73:223-231.
10. The EPIC investigators: Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty N Engl J Med 1994;330:956-961
11. Mølvig J, Baek L, Christensen P, Manogue KR, Vlassara H, Platz P, Nielsen LS, Svejgaard A, Nerup J. Endotoxin-stimulated human monocyte secretion of interleukin 1, tumor necrosis factor a, and prostaglandin E2 shows stable interindividual differences. Scand J Immunol. 1988;27:705-716
12. Santamaria P, Gherz RC, Bryan MK, Barbosa JJ. Involvement of class II MHC molecules in the LPs-induction of IL1/TNFsecretions by human monocytes: quantitative differences at the polymorphic level. J Immunol. 1989;143:913-922
13. Hayes MP, Zoon KC. Priming of human monocytes for enhanced lipopolysaccharide responses: expression of γ interferon, Interferon regulatory factors and tumor necrosis factor. Infect Immun. 1993;61:3222-322

9.2 Late lumen loss after coronary angioplasty is associated with the activation status of circulating phagocytes before treatment

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ABSTRACT

Background. The purpose of this pilot study was to identify biological risk factors for restenosis after percutaneous transluminal angioplasty (PTA) to predict the long-term outcome of PTA before treatment.

Methods and Results. To investigate whether blood granulocytes and monocytes could determine luminal renarrowing after PTA, several characteristics of these phagocytes were assessed before angioplasty in 32 patients who underwent PTA of one coronary artery and who had repeat angiograms at 6-month follow-up. The plasma levels of interleukin (IL)-1ß, tumor necrosis factor, IL-6, fibrinogen, C-reactive protein and lipoprotein(a) before angioplasty were assessed as well. We found that the expression of the membrane antigens CD64, CD66 and CD67 by granulocytes was inversely associated with the luminal renarrowing normalized for vessel size (relative loss) at 6 months after PTA, while the production of IL-1ß by stimulated monocytes was positively associated with the relative loss. Next, these univariate predictors were corrected for the established clinical risk factors of dilation of the left anterior descending coronary artery and current smoking, which were statistically significant classical predictors in our patient group. Only the expression of CD67 did not predict late lumen loss independent of these established clinical risk factors. Multiple linear regression analysis showed that luminal renarrowing could be predicted reliably (R^2=0.65; P<0.0001) in this patient group on the basis of the vessel dilated and only two biological risk factors that reflect the activation status of blood phagocytes, i.e., the expression of CD66 by granulocytes and the production of IL-1ß by stimulated monocytes.

Conclusions. The results of the present study indicate that activated blood granulocytes prevent luminal renarrowing after PTA, while activated blood monocytes promote late lumen loss. To validate this new finding further study in an independent patient group is required.

INTRODUCTION

Percutaneous transluminal coronary angioplasty (PTA) is a nonsurgical treatment of obstructive coronary artery disease (CAD), which, despite its high initial success rate of 90%, is compromised by the occurrence of restenosis (1). To increase the long term outcome of PTA the identification of risk factors for restenosis seems crucial.

The pathophysiology of restenosis has not yet been elucidated (2). It has been suggested that activated blood monocytes may contribute to this process by the production of cytokines and growth factors (1,3,4). The activation of granulocytes that occurs during PTA, through the release of proteinases and oxygen-derived free radicals, may have a potential bearing on the development of restenosis as well (5).

Circulating phagocytes are thought to reflect the *in vivo* state of the immune defense (6). The different phagocyte functions are mediated through specific membrane receptors. Members of the integrin class of adhesion receptors (CD11/CD18) function in the interaction of phagocytes with the endothelial lining (7), whereas receptors for the Fc moiety of IgG (CD16, CD32 and CD64) are involved in the pinocytosis of immune complexes and the phagocytosis of antibody coated particles (6). The expression of these membrane receptors can be stimulated by inflammatory mediators. For example, interferon gamma induces de novo expression of the high affinity Fc receptor CD64 on granulocytes (8). *N*-formylated peptides increase the expression of the receptor for activated complement C3bi (CD11b/CD18) on both granulocytes and monocytes (6). Activated granulocytes also express increased levels of CD66 and CD67 (9). In addition to the stimulated expression of membrane receptors, inflammatory mediators are capable of inducing a primed state in monocytes. In these primed cells, the stimulated production of cytokines is enhanced (10). Furthermore, stable, genetically-determined interindividual differences in the secretion of IL-1ß and tumor necrosis factor α (TNF) by lipopolysaccharide (LPS)-stimulated monocytes have been demonstrated (11,12).

To study if circulating granulocytes and monocytes might contribute to the process of restenosis, we investigated whether luminal renarrowing after PTA is associated with the activation status of circulating phagocytes.

METHODS

Patient group

Thirty-four patients gave informed consent for the study and were included according to the following criteria: 1) Stable or unstable angina pectoris (not refractory to medical treatment); 2) one culprit lesion responsible for the complaints; 3) successful PTA according to angiographic and clinical parameters (13). The mean age of the patients (26 men and 8 women) was 54.2 ± 8.3 years. The severity of the anginal complaints was classified according to the New York Heart Association, 8 patients were in class II, 17 were in class III, and 9 were in class IV. Nine patients had unstable angina (pain at rest and refractory to medical treatment). The target vessel was the left anterior descending coronary artery (LAD) in 17 patients, the left circumflex coronary artery (LCX) in 7 patients, and the right coronary artery (RCA) in 10 patients. Fifteen patients had suffered from myocardial infarction in the past, 6 patients had previously undergone PTA and in 2 patients coronary artery bypass grafting was performed before entrance in the study. The study population included 2 patients with diabetes mellitus, 22 patients with hypercholesterolemia (excluding 3 not known), and 21 patients with a family history of CAD. Sixteen patients were smokers: the 18 non-smokers included patients that had stopped smoking at least 6 months before PTA.

Protocol

Sheaths were inserted into the arterial and venous femoral vessels. To exclude any effect of day-to-day variation in the biological risk factors under study, 30 mL of blood was collected from the venous sheath immediately before treatment, anticoagulated with 0.2% EDTA, and kept at 4°C. Coronary angioplasty was performed with a steerable, movable guide-wire system via the femoral route. Details regarding the procedure used in our catheterization laboratory have previously been reported (13,14). Angiograms were obtained before and directly after PTA. Six months after the procedure, the patients were scheduled for follow-up angiography. The angiograms before and after PTA, and at 6-month follow-up were analyzed with the Coronary Angiographic Analysis System as described previously (13,14). When serious complaints of angina recurred before the 6 month follow-up, intercurrent coronary angiography was performed. If restenosis of the dilated segment was established PTA was repeated and the angiogram at that time (n=6 patients) was considered to be the end point for the study. Of the 34 patients who met the inclusion criteria, 32 repeat angiograms were obtained; 2 patients refused follow-up.

Luminal loss

The continuous variable luminal loss is defined as the change in minimal lumen diameter (MLD) during follow-up normalized for vessel size according to the following equation: relative loss (RLOSS) = ((postintervention MLD - follow-up MLD)/ vessel size) x 100%, and reflects the degree of luminal renarrowing. The vessel size is the value of the reference diameter function at the minimal position of the obstruction as previously described (14).

Reagents

The monoclonal antibodies (mAb) used in the flow cytometric analysis of membrane antigen expression were mAb B-B15 directed against CD11a, mAb 44 against CD11b, mAb 3.9 against CD11c, mAb B-H8 against CD15, mAb YFC 120.5 against CD16, mAb B-F1 against HLA-DR all obtained from Serotec (Oxford, UK). MAb IV.3 directed against CD32 and mAb 32.2 directed against CD64 were obtained from Medarex Inc (West Lebanon NH, USA). MAb CLB gran10 against CD66 and mAb B13.9 against CD67 were obtained from the Central Laboratory for Bloodtransfusion (Amsterdam, The Netherlands). MAb 5193 which recognizes HLA-DR_4 was from C-six Diagnostics Inc (Mequon, USA). The fluorescein conjugate of the hexapeptide *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys which is recognized by the receptor for *N*-formylated peptides on phagocytes was obtained from Molecular Probes (Eugene, OR, USA).

Flowcytometric analysis of membrane antigens

The binding of mAb to the cell surface was quantitated by fluorescence-activated cell sorting (FACStar, Becton Dickinson, Etten-Leur, The Netherlands). A buffy-coat fraction was obtained by centrifugation of the blood sample at 850g for 10 min. Contaminating erythrocytes were lysed in ammonium chloride solution consisting of 155 mM NH_4Cl, 10 mM $NaHCO_3$ and 0.1 mM EDTA. Next, leukocytes were washed with phosphate-buffered saline (PBS, pH 7.4) consisting of 140 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4.2H_2O$ and 2.7 mM KCl, centrifuged at 400g for 10 min and resuspended in PBS with 2% heat inactivated foetal calf serum (FCS) and NaN_3 (1 mg/mL). Leukocytes (4 x 10^5 cells) were incubated with mAb for 30 min. Leukocytes were washed four times and then incubated with fluorescein isothiocyanate (FITC) conjugated anti-mouse or anti-rat antibodies for 30 min after which the cells were washed four times and fixed with 1% paraformaldehyde in PBS with 2% FCS for 24 h at 4°C. Monocyte and granulocyte populations were separated by

gating on forward and perpendicular light scatter. Antigen expression is presented as specific linear fluorescence intensity (F.I.).

Cytokine release

To assess the capacity of monocytes to produce cytokines *in vitro*, monocytes were isolated from the buffy-coat fraction which was reconstituted with PBS to 20 mL, layered on top of a Lymphoprep gradient (Nycomed, Haarlem, The Netherlands), and centrifuged at 800g for 10 min at room temperature. The fraction containing the mononuclear leukocytes was washed with PBS. Next, the erythrocytes were lysed in ammonium chloride solution and the remaining mononuclear leukocytes were washed once with ammonium chloride solution, once with PBS and resuspended in RPMI (Flow Laboratories, Irvine, UK) with 2% FCS. The percentage of monocytes in this fraction was determined from cytospin preparations and in general amounted to 25%. Cells (6.3×10^5) were transferred into 96-wells culture plates (Costar, Badhoevedorp, The Netherlands) and incubated at 37°C in a humidified environment of 5% CO_2/ 95% air in the presence or absence of 5 ng/mL lipopolysaccharide (LPS), according to the method described by Endres et al. (15). After 24 hours supernatants were collected and stored at -70 °C until analysis of IL-1ß, IL-6 and TNF by an enzyme immuno assay (EIA, Medgenix, Amersfoort, The Netherlands). During this incubation period, cell viability as determined by trypan blue dye exclusion did not decrease significantly, and was > 95%. Cytokine release was expressed in pg per 10^4 monocytes.

Fibrinogen

High-and low-molecular-weight fibrinogen levels were determined with an EIA as previously described (16) and expressed in grams per liter.

C-Reactive protein

C-Reactive protein (CRP) was measured with an EIA (Dakopatts, Glostrup, Denmark) and expressed in milligrams per liter.

Lipoprotein(a)

The Lipoprotein(a) [LP(a)] concentration in plasma was determined with a radioimmunoassay (Pharmacia, Woerden, The Netherlands) and expressed in milligrams per liter.

Statistical analysis

The variation in duplicate measurements (intra-assay variation) did not exceed 10%. The strength of the association of late lumen loss with each of the potential biological risk factors described in the previous sections was assessed by linear regression analysis. Each new finding should be considered as suggestive, and needs validation in an independent patient group. No attempt was made to correct for multicomparisons, since it is not clear whether such a procedure is an improvement here (17,18). Since the expression of HLA-DR_4 is a characteristic cells may have or may not have, this variable is treated as a categorical one (no expression=0, expression=1). Each variable that proved to be statistically significant ($P<0.05$) in the univariate regression analysis was by multiple linear regression analysis to establish whether it was a risk factor independent of established clinical risk factors. The established risk factors included the vessel dilated ($P=0.001$; ß=0.5495) (LAD=1, other=0), and current smoking ($P=0.024$; ß=-0.3988) (yes=1, no=0). Other risk factors reported in literature were not statistically significant in the present study, i.e., family history of CAD ($P=0.072$; ß=0.3275), diabetes mellitus ($P=0.120$; ß=0.2807), hypercholesterolemia ($P=0.332$; ß=0.1867), unstable angina ($P=0.363$; ß=0.1665), hypertension ($P=0.569$; ß=0.1046), angina class ($P=0.674$; ß=0.0772) (class I/II=0, class III/IV=1), patient age ($P=0.826$; ß=0.0405) (age <65 years=0; ≥65 years=1) and sex ($P=0.882$; ß=0.0274). The new independent risk factors, together with the established clinical risk factors were used in a stepwise multiple linear regression analysis with P values for inclusion and elimination set at 0.05 and 0.10, respectively, to build a model that predicts the luminal renarrowing.

RESULTS

Variables predictive for late lumen loss

To investigate whether circulating phagocytes could contribute to luminal renarrowing after PTA, we determined the activation status of circulating phagocytes before treatment and assessed its association with the degree of luminal renarrowing (RLOSS) at 6 months after PTA. We identified several new univariate predictors of late lumen loss, the expression of CD64, CD66, and CD67 by granulocytes, and the stimulated production of IL-1ß by monocytes (Table 1a and 1b; Figs1 and 2). Next, the relative importance of these new univariate predictors with respect to established clinical risk factors was assessed. Multivariate regression analysis showed that three of the new univariate predictors were statistically significant independent predictors for luminal renarrowing after PTA (Table 1a

and 1b). CD66, an antigen expressed by granulocytes only, showed an inverse association with the relative luminal renarrowing at 6 months follow-up. This also was the case for the expression of CD64, the high-affinity receptor for immunoglobulin G (IgG). Furthermore, the RLOSS was positively associated with the amount of IL-1ß produced by monocytes in response to LPS. No other variables under study, including the plasma levels of LP(a), IL-1ß, TNF and fibrinogen, showed a significant relationship with late lumen loss (Table 1b).

TABLE 1a. CORRELATES OF LATE LUMEN LOSS: MEMBRANE ANTIGENS[a] EXPRESSED BY PHAGOCYTES

Variable	Univariate			Multivariate			
		regression coefficient			regression coefficient		
	P	b	β	P	b	β	n
Granulocytes							
CD66	0.004	-1.92	-0.4984	0.016	-1.44	-0.3760	32
CD67	0.014	-1.65	-0.4363	n.s.			31
CD64	0.029	-3.91	-0.3926	0.044	-3.11	-0.3125	31
CD11c	0.054	-2.50	-0.3434				32
fMLP[b]	0.075	-4.39	-0.3247				32
HLA-DR$_4$[c]	0.128	-11.69	-0.2795				30
CD16	0.133	0.11	0.3309				22
CD15	0.385	0.12	0.1589				32
CD11b	0.499	-0.22	-0.1240				32
CD32	0.539	0.18	0.1126				32
HLA-DR[c]	0.598	-0.32	-0.0985				30
CD11a	0.679	-0.17	-0.0761				32
Monocytes							
CD15	0.077	-1.14	-0.3174				32
fMLP[b]	0.117	-5.89	-0.2874				31
CD64	0.318	-0.22	-0.1824				32
HLA-DR$_4$[c]	0.361	-6.54	-0.1670				32
CD11a	0.417	0.12	0.1486				32
CD11b	0.561	-0.14	-0.1067				32
CD16	0.749	-0.15	-0.0722				22
CD11c	0.825	-0.08	-0.0406				32
CD32	0.948	-0.02	-0.0119				32
HLA-DR[c]	0.996	-0.00	-0.0010				32

[a]: CD = cluster of differentiation. Knapp et al. Leukocyte Typing IV. White cell differentiation antigens. Oxford: Oxford University Press, 1989.

[b]: The receptor for *N*-formylated peptides.

[c]: HLA-DR = human-leukocyte-associated antigens, class II type DR.

TABLE 1b. CORRELATES OF LATE LUMEN LOSS: INFLAMMATORY MEDIATORS AND LP(a)

Variable	Univariate			Multivariate			
		regression coefficient			regression coefficient		
	P	b	β	P	b	β	n
Produced by monocytes							
IL-1ß[a]	0.017	0.10	0.4415	0.025	0.08	0.3329	29
TNF[a]	0.080	0.14	0.3303				29
IL-6[a]	0.126	0.01	0.2908				29
IL-1ß[b]	0.165	0.07	0.2558				31
IL-6[b]	0.355	0.01	0.1721				31
TNF[b]	0.443	0.04	0.1430				31
Plasma levels of							
CRP	0.280	-0.74	-0.1969				32
TNF	0.501	0.25	0.1234				32
Fibrinogen	0.554	2.62	0.1086				32
IL-6	0.642	-0.17	-0.0854				32
IL-1ß	0.803	0.07	0.0458				32
LP(a)	0.965	<0.01	0.0080				32

[a]: Cytokine production of monocytes stimulated with 5 ng/mL LPS for 24 h.

[b]: Cytokine production of unstimulated monocytes.

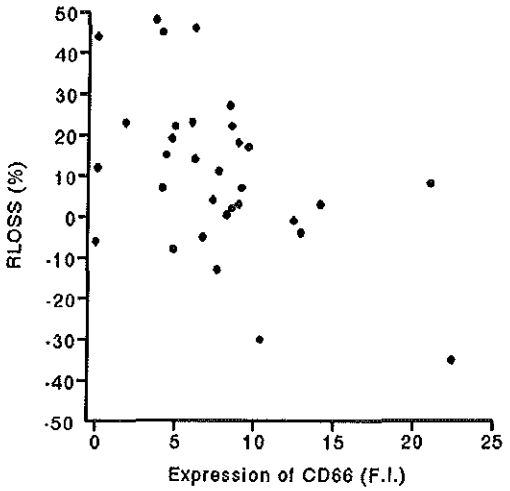

Fig 1. Association of the expression of the membrane antigen CD66 (F.I.) by blood granulocytes obtained from patients before PTA with the degree of luminal renarrowing expressed as relative loss (RLOSS) at six months after treatment, which can be described by the equation: $y = 25.25 - 1.92x$ ($R^2 = 0.25$; $P = 0.004$).

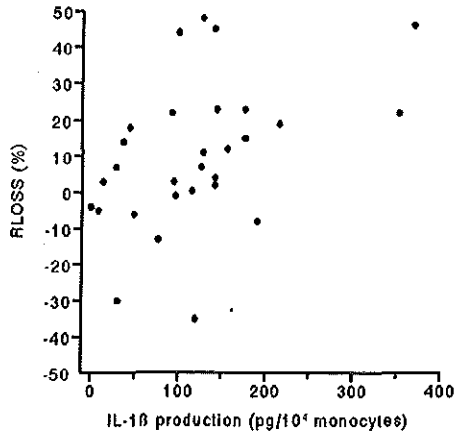

Fig 2. Association of the production of interleukin-1ß (pg/10^4 monocytes) by blood monocytes obtained from patients before PTA with the degree of luminal renarrowing expressed as relative loss (RLOSS) at six months after treatment, which can be described by the equation: $y = -2.34 + 0.10x$ ($R^2 = 0.19$; $P = 0.017$). The monocytes were stimulated by lipopolysaccharide (5 ng/mL) in vitro for 24 hr.

Multiple regression model to predict late lumen loss before PTA

To obtain a model that predicts the RLOSS before treatment, the relative contributions of the three new independent predictors of late lumen loss and the two established risk factors were analyzed by multiple regression using the stepwise procedure. This analysis showed that the RLOSS can be predicted from the expression of the activation marker CD66 (median: 7.8 F.I.; range: 0.0-22.4 F.I.) by granulocytes, the production of IL-1ß (median: 119.4 pg/10^4 monocytes; range: 0.0-368.9 pg/10^4 monocytes) by stimulated monocytes, and the vessel dilated (LAD vs RCA or LCX) according to the following equation:

RLOSS = 6.8 - 2.0 × (CD66) + 0.07 × (IL-1ß) + 17.3 × (LAD), (R^2=0.65; P<0.0001), with standard errors 6.3, 0.5, 0.03 and 4.8 for the constant and the regression coefficients, respectively. The standardized regression coefficients (ß) of CD66, IL-1ß and LAD amounted to -0.4671, 0.3055 and 0.4328, respectively.

Although fundamentally not correct but merely an attempt to show what could be expected of the model in an independent patient group, in Fig 3 the relationship between the predicted luminal diameter loss, calculated on the basis of this equation, and the observed relative loss is presented for each of our present patients. It is clear, however, that definitive conclusions about the predictive value of the model can only be obtained in an independent patient group.

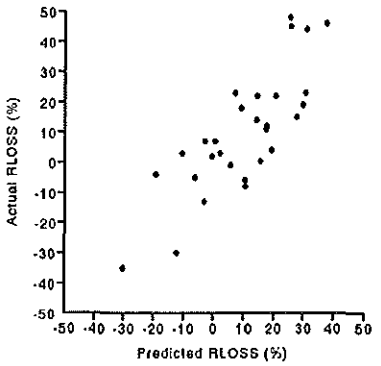

Fig 3. Relationship between the predicted degree of luminal renarrowing expressed as relative loss (RLOSS) at six months after treatment, calculated according to the equation (see also under Results): RLOSS = 6.8 - 2.0*(CD66) + 0.07*(IL-1ß) + 17.3*(LAD), and the observed RLOSS for each patient in our study group. Although this figure is fundamentally not correct, it is merely to illustrate the equation.

DISCUSSION

The major finding of the present study is that the activation status of phagocytes is associated with luminal renarrowing after PTA. Predictors for the degree of late lumen loss include characteristics of monocytes (IL-1ß production) and granulocytes (expression of CD64, and CD66). These new predictors and two established clinical risk factors (vessel dilated, and current smoking) were used to build a model by multiple linear regression analysis that predicts the RLOSS before treatment from 1) the production of IL-1ß by stimulated monocytes, 2) the expression of the activation marker CD66 by granulocytes, and 3) the vessel dilated (LAD vs. LCX and RCA).

The amount of IL-1ß a monocyte can secrete upon stimulation is genetically determined (11,12) and depends on the primed state of this type of phagocyte (10,19). It is assumed that IL-1 is an important determinant of intimal hyperplasia (20). *In vitro* studies have shown that IL-1 stimulates the thrombogenicity of EC and elevates levels of PDGF-A and PDGF-B chain transcripts in EC (21,22). Since PDGF has been shown to stimulate the migration of smooth muscle cells into the intima (23) this mechanism might be relevant. We found no relation between the degree of luminal renarrowing after PTA and the spontaneous release of IL-1ß by blood monocytes *in vitro* or the IL-1ß level of the plasma before treatment. However, the capacity of blood monocytes to synthesize IL-1ß upon stimulation *in vitro* was associated with the late outcome after PTA. This led us to hypothesize that the patients' blood monocytes in a response to vascular injury may infiltrate the lesion, become stimulated and depending on their capacity secrete IL-1ß that promotes intimal hyperplasia. Why only the stimulated production of IL-1ß, and not of IL-6 and of TNF, by monocytes is associated with relative lumen loss, is a matter of speculation. In the cascade model for restenosis Libby et al. (20) proposed that macrophages, which are a major source of IL-1, by the early acute cytokine generation evoke a secondary cytokine and growth factor response from other types of cell in the lesion including smooth muscle cells and endothelial cells that might establish a positive, self-stimulatory autocrine and paracrine feedback loop amplifying and sustaining the proliferative response.

We found an inverse association of relative luminal renarrowing with the expression of the high-affinity receptor for IgG (CD64) by granulocytes. Granulocytes express this antigen only after activation (8). However, CD64 did not significantly predict late lumen loss independent of the other multivariate predictors in our patient group. CD66, an antigen which is exclusively expressed by granulocytes, is considered to be an activation marker as well (9).

Recent evidence suggests that the CD66 antigens function as presenter molecules of the sialylated Lewis(x) antigen that bind to endothelial leukocyte adhesion molecule-1 expressed by activated EC (24). The important finding of the present study was that the degree of late lumen loss was low if the expression of CD66 by granulocytes was high. This suggests that activated granulocytes in fact could serve a protective role in the process of luminal renarrowing after PTA. In a way, this was an unexpected finding, since activated granulocytes are supposed to aggravate tissue damage by their potentially destructive armamentarium (5). However, the invasion of granulocytes at sites of injury is a normal response to injury which facilitates tissue repair. Could this be a likely initial response to PTA as well? A number of independent findings indeed point towards that direction. Firstly, in the cuffed rabbit carotid artery model of restenosis one of the earliest events is the infiltration of granulocytes into the lesion (25). The first 2-3 days of the inflammatory phase after PTA may be crucial for the ultimate outcome (3). Secondly, granulocytes relax human (internal mammary) artery (26). And finally, some years ago it was found that granulocytes could have a significant biological role in *preventing* thrombosis, rather than augmenting thrombosis, by the generation of 6-keto-prostaglandin-E_1 to inhibit platelet aggregation, and by providing 13-hydroxy-octadecadienoic acid in the absence of endothelial cells to inhibit platelet adhesion (27). Since local thrombosis after PTA might be a key event in the cascades of cytokine/growth factor production by macrophages and smooth muscle cells (20), a protective role of granulocytes could therefore be anticipated.

Recently it has been shown that granulocyte activation occurs directly after angioplasty (5). Our data indicate that the activation status of granulocytes *before* treatment may be crucial in *limiting* luminal renarrowing at 6 months after PTA.

Taken together, we conclude that the degree of late lumen loss might be predicted before treatment from the amount of IL-1ß produced by stimulated monocytes, the expression of CD66 by granulocytes, and the vessel treated by PTA. If our model reflects some major aspects of the process of luminal loss after PTA, interventional therapy could be directed to the function of granulocytes and monocytes. The failure of a multicenter trial conducted to determine if corticosteroids infused before PTA could reduce the rate of restenosis (28) indicates that broad spectrum drugs are not a first choice, and that timing to allow granulocytes to perform their beneficial role could be important. However, the model presented here first needs to be validated in an independent and larger patient group to establish its prognostic value.

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REFERENCES

1. Shirotani M, Yui Y, Kawai C. Restenosis after coronary angioplasty: Pathogenesis of neointimal thickening initiated by endothelial loss. *Endothelium.* 1993;1:5-22.
2. Schwartz RS, Edwards WD, Antoniades LC, Baiely KR, Holmes DR. Coronary restenosis: prospects for solution and new perspectives from a porcine model. *Mayo Clin Proc.* 1993;68:54-62.
3. Forrester JS, Fishbein M, Helfant R, Fagin J. A paradigm for restenosis based on cell biology: clues for the development of new preventive therapies. *J Am Coll Cardiol.* 1991;17:758-769.
4. Macdonald RG, Panush RS, Pepine CJ. Rationale for use of glucocorticoids in modification of restenosis after percutaneous transluminal coronary angioplasty. *Am J Cardiol.* 1987;60:56B-60B.
5. De Servi S, Mazzone A, Ricevuti G, Fioravanti A, Bramucci E, Angoli L, Specchia G. Granulocyte activation after coronary angioplasty in humans. *Circulation.* 1990;82:140-146.
6. Allen RC, Stevens DL. The circulating phagocyte reflects the in vivo state of immune defense. *Curr Opinion Infect Dis.* 1992;5:389-398.
7. Sluiter W, Pietersma A, Lamers JMJ, Koster JF. Leukocyte adhesion molecules on the vascular endothelium: Their role in the pathogenesis of cardiovascular disease and the mechnisms underlying their expression. *J Cardiovasc Pharmacol.* 1993;22:S37-S44.
8. Petroni KC, Shen L, Guyre PM. Modulation of human polymorphonuclear leukocyte IgG Fc receptors and Fc receptor mediated functions by IFN-gamma and glucocorticoids. *J Immunol.* 1988;140:3467-3472.
9. Tetteroo PAT, Bos MJE, Visser FJ, van dem Borne AEGKr. Neutrophil activation detected by monoclonal antibodies. *J Immunol.* 1986;136:3427-3432
10. Hayes MP, Zoon KC. Priming of human monocytes for enhanced lipopolysaccharide responses: expression of alpha interferon, interferon regulatory factors, and tumor necrosis factor. *Infection Immun.* 1993;61:3222-3227.
11. Mølvig J, Baek L, Christensen P, Manogue KR, Vlassara H, Platz P, Nielsen LS, Svejgaard A, Nerup J. Endotoxin-stimulated human monocyte secretion of interleukin 1, tumor necrosis factor alpha, and prostaglandin E_2 shows stable interindividual differences. *Scand J Immunol.* 1988;27:705-716.
12. Santamaria P, Gehrz RC, Bryan MK, Barbosa JJ. Involvement of class II MHC molecules in the LPS-induction of IL-1/TNF secretions by human monocytes. Quantitative differences at the polymorphic level. *J Immunol.* 1989;143:913-922.
13. Hermans WR, Rensing BJ, Foley DP, Tijssen JGP, Rutsch W, Emanuelsson H, Danchin N, Wijns W, Chappuis F, Serruys PW. Patient, lesion and procedural variables as risk factors for luminal re-narrowing after successful coronary angioplasty: a quantitative analysis in 653 patients with 778 lesions. *J Cardiovasc Pharmacol.* 22(Suppl 4);S45-S57.
14. Reiber JHC, Serruys PW, Kooijman CJ, Wijns W, Slager CJ, Gerbrands JJ, Schuurbiers JHC, den Boer A, Hugenholtz PG. Assessment of short-, medium- and longterm variations in arterial

dimensions from computer assisted quantitation of coronary angiograms. *Circulation.* 1985;71:280-288.

15. Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonneman G, van der Meer JWM, Cannon JG, Rogers TS, Klempner MS, Weber PC, Schaefer EJ, Wolff S, Dinarello CA. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *N Engl J Med.* 1989;320:265-271.
16. Hoegee-de Nobel E, Voskuilen M, Briët E, Brommer EJP, Nieuwenhuizen W. A monoclonal antibody-based quantitative enzyme immunoassay for the determination of plasma fibrinogen concentrations. *Thromb Haemostas.* 1988;60:415-418.
17. Miettinen OS. *Theoretical Epidemiology. Principles of Occurrence Research in Medicine.* New York: Wiley and Sons; 1985:114-116.
18. Rothman KJ. *Modern Epidemiology.* Boston: Little, Brown and Company; 1986:147-151.
19. Rola-Pleszczynski M, Lemaire I. Leukotrienes augment interleukin 1 production by human monocytes. *J Immunol.* 1985;135:3958-3961.
20. Libby P, Schwartz D, Brogi E, Tanaka H, Clinton SK. A cascade model for restenosis. A special case of atherosclerosis progression. *Circulation.* 1992;86[suppl III]:III47-III52.
21. Bevilaqua MP, Schleef RR, Gimbrone MA, Loskutoff DJ. Regulation of the fibrinolytic system of cultured human vascular endothelium by interleukin-1. *J Clin Invest.* 1986;78:587-591.
22. Gay CG, Winkles JA. Heparin-binding growth factor-1 stimulation of endothelial cells induces platelet derived growth factor A-chain gene expression. *J Biol Chem.* 1990;265:3284-3292.
23. Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, Clowes AW. Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. *J Clin Invest.* 1992;89:507-511.
24. Kuijpers TW, Hoogerwerf M, van der Laan LJ, Nagel G, ván der Schoot CE, Grunert F, Roos D. CD66 nonspecific cross-reacting antigens are involved in neutrophil adherence to cytokine-activated endothelial cells. *J Cell Biol.* 1992;118:457-466.
25. Kockx MM, De Meyer GRY, Jacob WA, Bult H, Herman AG. Triphasic sequence of neointimal formation in the cuffed carotid artery of the rabbit. *Artheriosclerosis Thromb.* 1992;12:1447-1457.
26. Mehta JL, Malhotra S, Crocker B, Lawson DL, Martin TD, Mayfield WR, Staples ED. Polymorhonuclear leukocytes relax human internal mammary artery and saphenous vein segments differently. *Cor Art Dis.* 1991;2:379-388.
27. Buchanan MR. Inhibition of thrombosis by leukocytes: role of endogenous fatty acid metabolites. *Sanofi Foundation Thromb Res.* 1989;4:14-22.
28. Pepine CJ, Hirshfeld JW, Macdonald RG, Henderson MA, Bass TA, Goldberg S, Savage MP, Vetrovec G, Cowley M, Taussig AS, Whitworth HB, Margolis JR, Hill JA, Bove AA, Jugo R, for the M-HEART Group. A controlled trial of corticoids to prevent restenosis after coronary angioplasty. *Circulation.* 1990;81:1753-1761.

10 General Discussion

In order to interfere with the accumulation of chronic inflammatory cells in atherosclerosis, and allied problems like restenosis after PTA, it is of importance to define the intracellular signalling mechanisms underlying the endothelial expression of VCAM-1. In the present thesis we have studied the expression of VCAM-1 in a model of human umbilical vein endothelial cells stimulated with TNF. The experimental work described here was focussed on the hypothetical role of reactive oxygen species, and the p38 MAPK pathway in the intracellular signalling elicited by TNF. We have also investigated whether luminal renarrowing after PTA of a coronary artery in patients is associated with the activation status of blood monocytes. Along this line of investigation we have studied the possible relation of restenosis with the activation status of circulating granulocytes as well. The results of the patient and experimental studies are discussed here in five separate paragraphs.

Late lumen renarrowing is associated with the activation status of circulating phagocytes before treatment

Restenosis after PTA develops in 20 to 40 % of the cases, suggesting that patient characteristics determine the ultimate outcome of this treatment. To improve the longterm outcome the identification of risk factors for restenosis is crucial. We have demonstrated in chapter 9.2 that in patients undergoing PTA of a coronary vessel luminal renarrowing is associated with the activation status of circulating phagocytes before treatment. The finding that the potential amount of interleukin 1β (IL-1) produced by activated monocytes is positively associated with the extent of luminal renarrowing in patients suggests that circulating monocytes accumulate at the PTA site, and contribute to the process of restenosis. This is in line with experimental evidence by Schwarz et al., who reported that the normal porcine artery responds to severe mechanical injury in three distinct sequential phases, which include the recruitment of circulating monocytes (1). Just after local thrombus formation there is a cellular recruitment phase in which monocytes/macrophages line the lumen surface, and migrate into a degenerating thrombus beneath the newly formed endothelium (1). This process is likely mediated through the expression of VCAM-1 by the remaining endothelial cells as shown by Tanaka in experimental animals (2). Libby *et al* proposed that inside the lesion monocytes contribute to the smooth muscle cell proliferation and matrix accumulation through the synthesis of the cytokines IL-1 and TNF (3). IL-1 and TNF have similar activities on endothelial cells. Both IL-1 and TNF promote the expression of the endothelial cell

adhesion molecules (chapter three and four), and stimulate tissue factor procoagulant activity (4,5). However, despite the overlapping activities the results presented in chapter nine of the thesis show that the potential production of IL-1 by LPS-stimulated monocytes is a more important determinant of the outcome of the PTCA treatment than that of TNF. A possible explanation might be that TNF also stimulates programmed cell death or apoptosis in certain sensitive cell types for example T-lymphocytes (6), which in comparison to IL1 could inhibit the cellular aspect of restenosis. However, this is highly speculative since it remains to be determined whether the *in vitro* activities of TNF can be extrapolated to the *in vivo* situation. Also it remains to be demonstrated whether the potential (*in vitro*) production of cytokines reflects the *in vivo* activity of monocytes.

An unexpected finding of this study was the inverse association of luminal renarrowing with the activation status of circulating granulocytes reflected by a high expression of the activation marker CD66. Granulocytes express four different CD66 family members, i.e., CD66a, CD66b (formerly known as CD67), CD66c, CD66d. The CD66 mAb Gran10 used in our study is recognizes CD66a,CD66c, and CD66d on granulocytes, whereas the so called CD67 mAb B13.9 used here in fact recognizes CD66b (7). Despite structural differences CD66a, CD66b, and CD66c, have all been found to associate intracellularly with tyrosine kinases like Lyn and Hck (7). This tyrosine kinase activity may be involved in signal transduction via the CD66 family members. This is relevant since it has been demonstrated that CD66 family members (except CD66b) expressed by granulocytes function as presenter molecules for the sialylated Lewis^X antigen (8), which binds the endothelial adhesion molecules E- and P-selectin facilitating the increased rolling of granulocytes. Upon binding of E-selectin granulocytes become activated, possibly through signals emanating form the CD66 receptor, and firmly adhere to ICAM-1. Therefore our data suggest that, like monocytes, granulocytes accumulate at the site of tissue injury after PTA using their CD66 receptor. The mechanism underlying the protective action of activated granulocytes in restenosis after PTCA remains to be elucidated. It is worth noting that granulocytes can produce 6-keto-prostaglandin E_1, which inhibits platelet aggregation, and provide 13-hydroxy-octadecadienoic acid which inhibits platelet adhesion. In this way granulocytes could prevent thrombosis a key event in the response to tissue injury after PTA (9, chapter 9.1). Although it has been known for some time that granulocyte activation occurs in response to the PTCA treatment (10,11) our results suggests for the first time that granulocytes may have a protective effect in the development of restenosis.

Recently our findings have been debated by Mickelson *et al.* (12). In a study group of eleven patients they found that expression of CD11b on granulocytes was *positively* associated with the clinical outcome of the PTA. In other words the higher the CD11b expression the more chance of experiencing late clinical events including coronary artery bypass grafting (three patients), myocardial infarction (one patient), and unstable angina (two patients). We did not find an association between CD11b expression and the PTA outcome. This is particularly confusing since both the CD66 and CD11b antigens appear to be stored in the same specific granules (13). Mickelson *et al.* have argued that our results may be attributed to activation of the granulocytes due to preparative procedures (12). However, it is very unlikely that we would have observed any relation at all if the granulocytes were already maximally stimulated by the preparative procedures. Therefore the contradictory results cannot simply be attributed to the preparative procedures supposedly activating the granulocytes of all patients to the same level. What could be relevant here is that both studies use different endpoints to determine restenosis. While we used a continuous variable to quantify the extent of luminal renarrowing Mickelson *et al* used a dichotomous variable and therefore risk the chance of misclassification of some of their patients. Especially with a small number of subjects this may lead to erroneous results. We have to await validation in a larger study before definitive conclusions can be drawn.

Taken together we have presented evidence that in patients undergoing PTA of a coronary artery the extent of luminal renarrowing depends upon the activation status of circulating granulocytes and monocytes. The results of our study implicate that the outcome of PTA can be favorably modulated if the activity of circulating granulocytes is stimulated and the activation of monocytes is inhibited at the time of PTA.

Reactive oxygen species and the expression of VCAM-1 on TNFα-stimulated endothelial cells

In order to interfere with the accumulation of circulating monocytes it is of importance to elucidate the intracellular signalling mechanism involved in the expression of VCAM-1. In the past five to ten years a number of second messenger molecules and proteins have been identified that are involved in the intracellular signalling of TNF. A novel class of second messengers may be the oxygen centred radicals or reactive oxygen species (ROS). The experimental studies described in the present thesis aimed at investigating the possible role of ROS in the expression of VCAM-1, since this would be a new intracellular signalling mechanism next to protein phosphorylation, the cyclic nucleotide system, and signalling

through the inositol phosphates and intracellular calcium. Different approaches were used and the results will be discussed here.

Cytochrome P450 is the oxygen dependent component in the expression of VCAM-1

In the first place the effect of hypoxia on the expression of VCAM-1 was studied and compared with that on the expression of E-selectin and ICAM-1. The results presented in chapter four show that hypoxia specifically inhibited the expression of VCAM-1 to about 60% of the control. In contrast hypoxia did not have any effect on the expression of E-selectin or ICAM-1. These findings are in accordance with those of Klein *et al.*, who demonstrated a slightly larger decrease of VCAM-1 expression under hypoxic conditions (14). The oxygen dependence of VCAM-1 expression was investigated more closely. Inhibitors of the lipoxygenase and cyclooxygenase did not inhibit VCAM-1 expression (Table I).

Table I: TNF induced VCAM-1 expression in the presence of lipoxygenase and cyclooxygenase inhibitors

	Fluorescence intensity ± standard error
Control	43 ± 13
Nordihydroguaiuretic acid	35 ± 6
MK866	42 ± 4
Indomethacine	41 ± 8

Endothelial cells were preincubated with nordihydroguaiuretic acid (10 µM, MK866 (10 µM) or indomethacin (10 µM) for 30 min, and subsequently stimulated with TNF in the presence of these agents. At 6 h after stimulation VCAM-1 expression was assessed by FACS-analysis. The results are averaged of three separate experiments.

The contribution of another candidate enzyme, i.e., the phagocyte type NADPH oxidase, was investigated next. The NADPH-oxidase inhibitor apocynin blocks VCAM-1 expression at the transcriptional level, but has no effect on the expression of ICAM-1. Indicating that a phagocyte type NADPH oxidase may be involved in the endothelial expression of VCAM-1. Western blotting of endothelial cell homogenates showed no expression of the $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$ proteins in endothelial cells. However, Jones et al have recently demonstrated the expression of the messenger RNA for these proteins by the more sensitive RT-PCR technique, and immunolabeling techniques ($p67^{phox}$) (15). Still these authors also

conclude that no functional phagocyte type NADPH oxidase is present in the endothelial cells because heme spectroscopy failed to indicate the presence of the low-potential cytochrome b558, an essential component of the NADPH-oxidase (15). Furthermore apocynin does not solely inhibit the NADPH-oxidase of phagocytes. We show in chapter six that apocynin also inhibits cytochrome P450 monooxygenase activity present in endothelial cells, by measuring the dealkylation of cytochrome P450 IA1 substrate 7-ethoxyresorufin. This is in line with data reported by Swart *et al* who showed that apocynin inhibits cytochrome P450 activity in sheep adrenal cells (16). Taken together the absence of a functional NADPH-oxidase in endothelial cells, the inhibition of VCAM-1 expression by apocynin and by hypoxia, and the inhibition of cytochrome P450 by apocynin suggest that the cytochrome P450 monooxygenase is involved in the TNF-stimulated endothelial expression of VCAM-1. Whether this finding can be considered as proof for a role of ROS will be discussed next.

Second messengers involved in the expression of VCAM-1: ROS or lipid

Monolayers of unstimulated endothelial cells secrete superoxide anions into the medium (17,18,19). The extracellular concentration of these superoxide anions can be increased by TNF, IL1, and IFNγ (18,19). Although this may support a role for the superoxide anion as an second messenger, an increased intracellular concentration of superoxide after stimulation with TNF remains to be demonstrated as yet.

The superoxide anion may not be directly involved as an intracellular second messenger in the activation of NFκB. In mammary carcinoma cells (MCF-7) increased inactivation of the superoxide anion, mediated by overexpression of the Mn-superoxide dismutase, does not impair the activation of NFκB (20). Hydrogen peroxide, on the other hand, directly activates NFκB in Jurkat T-cells (20). Therefore we have studied whether TNF stimulates the production of hydrogen peroxide in endothelial cells. Hydrogen peroxide readily passes the (endothelial) cell membrane. Therefore the extracellular release of hydrogen peroxide reflects its intracellular production (21). We found that the basal extracellular release of hydrogen peroxide of 33 pmol/ min/ mg protein (or 1.3 pmol/min /10^5 cells) did not increase after stimulation of the endothelial cell with 100 U/ml TNF. These data were in agreement with Royall who reported a release of 75 pmol/ min/ mg protein in bovine aortic endothelial cells and no effect of TNF at 1000 U/ml. These results seem to be in contrast with other studies in which TNF (19), and also IL1 and IFNγ (18), stimulate the basal extracellular release of superoxide anions about three to fourfold. Differences in culture conditions may have

attributed to these different results. Whereas Weber *et al* and Matsubara *et al* respectively used detached endothelial cells or subconfluent monolayers, we used confluent monolayers of endothelial cells. Another possible explanation may be that superoxide not only dismutates into hydrogen peroxide but reacts with other molecules as well. For instance superoxide could react with nitric oxide and form peroxynitrite. However, peroxynitrite is a powerful oxidant that has been linked to disease processes in nerve cells (22). Therefore under non-pathological conditions the simultaneous production of superoxide and nitric oxide supposedly is minimal. Unfortunately, it was technically not feasible to precisely determine the *intracellular* concentration of superoxide. Therefore a possible role for the superoxide anion as second messenger molecule in endothelial cells stimulated with TNF can not definitively be excluded here.

Theoretically an increased intracellular concentration of ROS could have induced an increase in lipid peroxidation and/ or an increased activity of the pentose phosphate pathway. To investigate whether TNF increases lipid peroxidation in endothelial cells we used the fluorescent cis-parinaric acid that is oxidized in the presence of peroxides. The results presented in chapter eight show that TNF does not increase lipid peroxidation in endothelial cells. Furthermore the activity of the pentose phosphate pathway did not increase after stimulation with TNF. Whereas a positive control of 20 μM cumene hydroperoxide stimulated the production of $^{14}CO_2$ from 1-^{14}C-labeled glucose about four times (data not shown). The evidence presented here leads us to the conclusion that hydrogen peroxide does not play a role as second messenger molecule in the TNF stimulated expression of VCAM-1 by endothelial cells.

In addition to hydrogen peroxide a wide variety of structurally diverse compounds, e.g., steroids, fatty acids (including prostaglandins and leukotrienes) are metabolized by the cytochrome p450 monooxygenase. For example arachidonic acid (AA) is metabolized by the P450 family members AA ω/ω-oxygenase and AA-epoxygenase (23,24). AA is generated from glycerophospholipids by PLA_2. When this source of second messenger is turned off by the PLA_2 inhibitor bromophenacylbromide (chapter six) VCAM-1 but not ICAM-1 expression is significantly decreased. Although this may indicate that an AA metabolite is involved in the VCAM-1 expression by endothelial cells upon stimulation with TNF the cytochrome P450 family member, the nature of the second messenger involved still has to be elucidated.

Since cytochrome P450 is specifically involved in the expression of VCAM-1, it seems unlikely that the cytochrome P450 monooxygenase is involved in the activation of NFκ-B-binding elements, which are present in the promotors of VCAM-1 as well as ICAM-1 and E-selectin. In contrast to NFκ-B, an IRF-1 binding element is present only in the promotor of VCAM-1. Activation of the interferon-stimulated response elements (ISRE) has previously been shown to involve signal transduction through metabolism of AA (25).

The role of iron in the regulation of VCAM-1 expression

Despite the fact that TNF did not increase the production of hydrogen peroxide, ROS still may contribute to the intracellular signalling of TNF in a different way. In the presence of hydrogen peroxide an increase in the low molecular weight (LMW) iron pool could lead to the enhanced formation of the highly reactive hydroxyl radical. Therefore the LMW iron pool was quantified with the fluorescent probe calcein according to the method described by Epsztejn *et al* (26). The results presented in chapter eight show that the LMW iron pool of 2.3 ± 1.8 μM does not change after stimulating endothelial cells with TNF. Therefore changes in the intracellular concentration of the hydroxyl radical are also not anticipated, which makes a signalling function for the hydroxyl radical and the LMW iron pool in the expression of VCAM-1 rather unlikely. However, when endothelial cells are stimulated in the presence of the iron chelator desferrioxamine VCAM-1 expression was reduced to about 50% (chapter eigth), leaving the ICAM-1 expression intact. Considering the results presented above this must not be interpreted as proof for a role of the LMW pool in the expression of VCAM-1. We found that in addition to chelating the LMW pool, desferrioxamine also chelates protein-bound iron. Already after 30 min of incubation with desferrioxamine the endothelial aconitase activity was decreased to 40% of the control. We therefore conclude that iron in a protein bound form specifically regulates the expression of VCAM-1.

We have further investigated this and demonstrated that desferrioxamine does not affect the accumulation of messenger RNA for VCAM-1. To investigate whether VCAM-1 translation involves an iron containing enzyme, the incorporation of ^{35}S-methionine into immunoprecipitated VCAM-1 was assessed. Desferrioxamine reduced the incorporation of ^{35}S-methionine to about 50%. Therefore in addition to the transcriptional regulation, VCAM-1 expression also involves translational control mechanisms which requires the presence of an iron dependent enzyme.

P38 MAP kinase is involved in the post-transcriptional regulation of VCAM-1

In addition to the results with the iron chelator desferrioxamine more evidence was accumulated that VCAM-1 expression is regulated at the post-transcriptional level. The p38 MAPK may play an important part in this regulatory process.

The p38 MAPK can be activated by oxidative stress (27) and cytokines, like TNF. P38 MAPK probably has a distinct function in the cell. Its activity has been linked to the phosphorylation and activation of the transcription factor ATF-2, and the MAPK-activated protein kinase-2 (MAPKAPK-2), which on its turn phosphorylates the small heat shock proteins (Hsp's) Hsp25 and Hsp27 (chapter seven)

To investigate whether the p38 MAPK cascade is functional in endothelial cells MAPKAPK-2 activity was assessed in these cells following stimulation with TNF. The results showed that endothelial cells exhibit a basal MAPKAPK-2 activity which is increased about three fold by TNF. The increased MAPKAPK-2 activity is accompanied by a similar increase in the phosphorylation of endogenous Hsp27 (chapter seven). Next, the contribution of the p38 MAPK was assessed by using the specific p38 MAPK inhibitor SB203580 (28). SB203580 inhibits p38 MAPK in the μM range and does not affect the activity of a number of other kinase and phosphatases, e.g., MAPKAP-2, JNK, ERK2, protein phosphatases-1 and 2A (28). Our data demonstrated that the expression of VCAM-1 at the cellular membrane was reduced to about 50% of the control, whereas the expression of ICAM-1 remained unaffected. Recently, it has been demonstrated that p38 also is involved in the transcriptional activation of E-selectin. However, in contrast to E-selectin the VCAM-1 promotor does not contain the consensus cAMP responsive element/ activating transcription factor element (CRE/ATF) sequence TGACGTCA, nor the TGACATCA present in the E-selectin promotor. In fact inhibition of the p38 MAPK had no effect on the accumulation of VCAM-1 messenger RNA. We therefore conclude that p38 MAPK is involved in the post-transcriptional regulation of VCAM-1 expression. Whether this control is exerted at the translational level remains to be determined. In this respect it is of interest to note that the regulation of TNF translation is mediated through an AUUUA repeated motif located in its 3'UTR (30). Deletion of this region from the GM-CSF mRNA led to a great increase of its translation efficiency and in the case of TNF mRNA to a constitutive synthesis of TNF protein (31,30). These AUUUA regions, which are also present in the 3'UTR of the messenger for VCAM-1, have been implicated in the regulation of mRNA stability and specifically bind 37-40 kDa proteins (32). Lee and Young have postulated that the translational control of TNF biosynthesis involves an inactive translational complex of proteins bound to the AUUUA repeated motif in the 3'UTR

which has folded back onto the 5'UTR of the TNF messenger. Phosphorylation of this complex by the p38 MAPK would relieve the translational repression (33). Whether this model depicted in Fig. 1 applies to the translational regulation of VCAM-1 is an area open to further investigation.

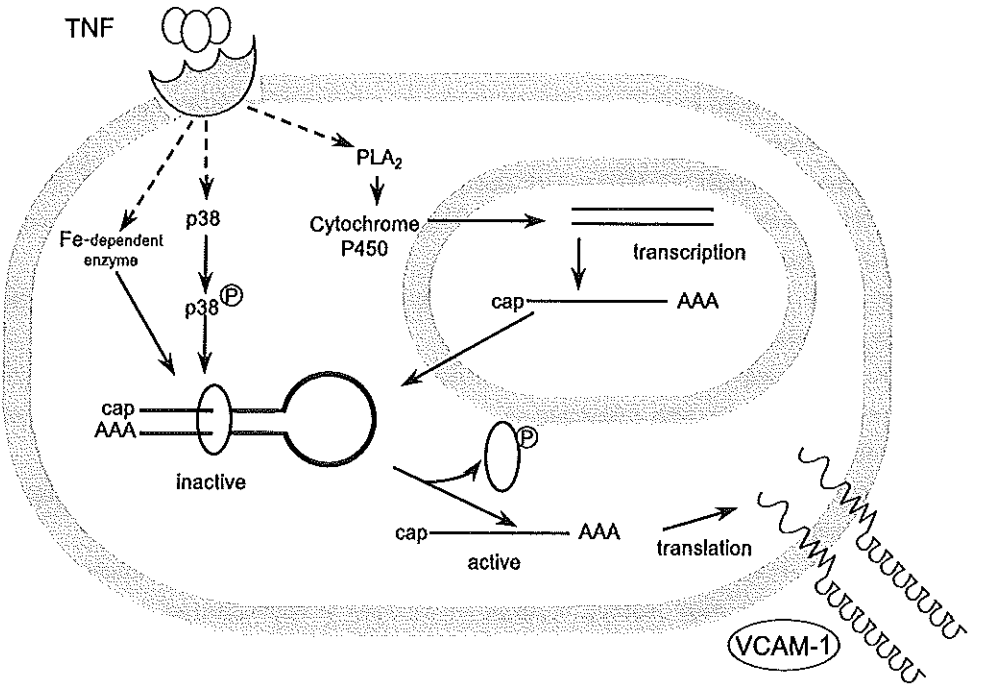

Fig 1. Schematical and hypothetical representation of the intracellular signalling pathways involved in the TNF-stimulated endothelial expression of VCAM-1. Modified from reference 33.

Conclusion

The expression of VCAM-1 on TNF stimulated endothelial cells is subject to regulation through specific intracellular signalling cascades. Previously the effects of antioxidants on the stimulated expression of VCAM-1 have been used as proof for a role of oxygen centred radicals as second messenger molecules in transcription regulation. In the present thesis we have shown that the oxygen dependent step presumably is catalyzed by the cytochrome P450 monooxygenase. A closer investigation on the p38 MAPK pathway and the effects of the iron chelator desferrioxamine in endothelial cells reveals that VCAM-1 is also specifically regulated at the translational level. Whether the specific regulation of VCAM-1 expression may be used to prevent the accumulation of circulating monocytes in the treatment of restenosis after PTA remains to be demonstrated.

References

1. Schwartz RS, Holmes DR, Topol EJ. The restenosis paradigm revisited: an alternative proposal for cellular mechanisms. J Am Coll Cardiol. 1992;20:1284-1293
2. Tanaka H, Sukhova GK, Swanson SJ, Clinton SK, Ganz P, Cybulsky MI, Libby P. Sustained activation of vascular cells and leukocytes in the rabbit aorta after balloon injury. Circulation 1993;88:1788-1803
3. Libby P, Schwartz D, Brogi E, Tanaka H, Clinton S. A Cascade model for restenosis. A special case of atherosclerosis progression. Circulation 1992;86:III-47-III-52
4. Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone MA. Interleukin-1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells J Ex Med 1984;160:618-623
5. Nawroth PP, Stern DM. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J Ex Med 1986;163:740-745
6. Hernandez-Caselles T, Stutman O. Immune functions of TNF. 1. TNF induces apoptosis of mouse thymocytes and can also stimulated or inhibit IL-6 induced proliferation depending on the concentration of mitogenic costimulation. J Immunol 1993;151:3999-4012
7. Skubitz KM, Campbell KD, Ahmed K, Skubitz APN. CD66 family members are associated with tyrosine kinase activity in human neutrophils. J Immunol 1995;155:5382-5390.
8. Kuijpers TW, Hoogerwerf M, vander Laan LJW, Nagel G, van der Schoot CE, Grunert F, Roos D. CD66 nonspecific cross reacting antigens are involved in neutrophil; adherence to cytokine-activated endothelial cells. J Cell Biol. 1992;118:457-466
9. The EPIC investigators: Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty N Engl J Med 199433:956-961
10. DeServi S, Mazzone, Ricevuti G *et al.* Granulocyte activation after coronary angioplasty in humans. Circulation 1990;82:140-146
11. Ikeda H, Nakayama H, Oda T *et al.* Neutrophil activation after percutaneous transluminal coronary angioplasty. Am Heart J 1994;82:1091-1098
12. Mickelson JK, Lakkis NM, Villarreal-Levy G, Hughes BJ, Smith CW. Leukocyte activation with platelet adhesion after coronary angioplasty: a mechanism for recurrent disease? JACC 1996;28:345-353
13. Tetteroo PAT, Bos MJE, Visser FJ, von dem Borne. Neutrophil activation detected by monoclonal antibodies. J Immunol 1986;136:3427-3432
14. Klein CL, Kohler H, Bittinger F, Otto M, Hermanns I, Kirkpatrick CJ. Comparative studies on vascular endotheliumin vitro 2. Hypoxia: Its influences on endothelial cell proliferation and expression of adhesion molecules. Pathobiology 1995;63:1-8
15. Jones SA, O'Donnell VA, Wood JD, Broughton JP, Hughes EJ, Jones OTG. Expression of phagocyte NADPH oxidase components in human endothelial cells. Am J Physiol 1996;271: H1626-1634

16. Swart P, van der Merwe KJ, Swart AC, Todres PC, Hofmeyr JHS. Inhibition of cytochrome P-$450_{11\beta}$ by some naturally occurring acetophenones and plant extracts from the shrub *Salsola tuberculatiformis*. Planta Medica 1993;59:139-143
17. Mohazzab-H KM, Kaminski PM, Wolin MS. NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. Am J Physiol (Heart Circ Physiol) 1994;266:H2568-H2572
18. Matsubara T, Ziff M. Increased superoxide anion release from human endothelial cells in response to cytokines. J Immunol 1986;137:3295-3298
19. Weber C, Erl W, Pietsch A, Ströbel M, Ziegler-Heitbrock HWL, Weber PC. Antioxidants inhibit monocyte adhesion by suppressing nuclear factor κB mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. Arterioscl Thromb 1994;14:1665-1673
20. Schreck R, Meier B, Männel DN, Dröge Baeuerle PA. Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. J Ex Med 1992;175:1181-1194
21. Royall JA, Gwin PD, Parks DA, Freeman BA. Responses of vascular endothelial oxidant metabolism to lipopolysaccharide and tumor necrosis factor-α. Arch Biochem Biophys 1992;294:686-694
22. Butler AR, Flitney FW, Williams DLH. NO, nitrosonium ions, nitroxide, nitrothiols and iron-nitrosyls in biology a chemist's perspective. TIPS 1995;16:18-22
23. McGiff JC, Steinberg M, Quilley J. Missing links: cytochrome P450 arachidonate products. A new class of lipid mediators. Trends Cardiovasc Med 1990;6:4-10
24. Fitzpatrick FA, Murphy RC. Cytochrome P450 metabolism of arachidonic acid: formation and biological actions of 'epoxygenase'-derived eicosanoids. Pharmacol Rev 1989;40:229-241
25. Hannigan GE, Williams BRG. Signal transduction by interferon α through arachidonic acid metabolism. Science 1991;251:204-207
26. Epsztejn S, Kakhlon O, Glickstein H, Breuer W, Cabantchik ZI. Fluorescence of the labile pool of mammalian cells. Anal Biochem 1997;248:31-40
27. Huot J, Houle F, Marceau F, Landry J. Oxidative stress-induced actin reorganization mediated by the p38 mitogen activated protein kinase/ heat shock protein 27 pathway in vascular endothelial cells. Circ Res 1997;80:383-392
28. Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses by cellular stresses and interleukin-1. FEBS Letters 1995;364:229-233
29. Read MA, Whitley MZ, Gupta S, Pierce JW, Best J, Davis RJ, Collins T. Tumor necrosis factor α-induced E-selectin expression is activated by the nuclear factor-κB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways. J Biol Chem 1997;272:2753-2761
30. Han J, Huez G, Beutler B. Interactive effects of the tumor necrosis factor promotor and 3'-untranslated regions. J Biol Chem 1991;146:1843-1848
31. Kruys V, Marinx O, Shaw G, Deschamps J, Huez G. Translational blockade imposed by cytokine-derived UA-rich sequences. Science 1989;245:852-854

32. Zubiaga AM, Belasco JG, Greenberg ME. The nonamer is the key AU-rich sequence motif that mediates mRNA degradation. Mol Cell Biol. 1995;15:2219-2230
33. Lee JC, Young PR. Role of CSBP/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. J Leuk Biol1996;59:152-1

Summary

The accumulation of leukocytes into tissues characteristically occurs in several distinct steps. Firstly, leukocytes roll along the vessel wall through the formation of reversible bonds between sialylated Lewis^x and Lewis^A antigens on the leukocytes and P- and E-selectins on the endothelial cells. In close proximity to the vessel wall the leukocytes become activated, and firmly adhere to the endothelial cells of the vasculature. This firm adhesion is mediated through integrin molecules expressed by leukocytes, and the immunoglobulin-like adhesion molecules VCAM-1 and ICAM-1 expressed by endothelial cells. Finally, the adherent leukocytes migrate into the tissue in the direction of a chemotactic gradient.

In contrast to ICAM-1, VCAM-1 specifically mediates the accumulation of circulating monocytes, which play an important role in chronic inflammatory reactions. Recently, it has become clear that in addition to mediating firm adhesion, VCAM-1 can also trigger rolling of circulating monocytes along the vessel wall. Therefore firm adhesion of monocytes can also occur in the absence of simultaneous selectin expression. The present thesis focusses on VCAM-1.

Under normal, non-inflammatory, conditions endothelial cells do not express VCAM-1. After stimulation with the inflammatory cytokine TNF VCAM-1 appears on the endothelial cell membrane. ICAM-1 is already present under normal conditions, and upregulated by stimulation with TNF. The expression of both VCAM-1 and ICAM-1 lasts for 24- 48 hours. A striking difference between these adhesion molecules is that the expression of VCAM-1, but not the expression of ICAM-1, can be inhibited by antioxidants. From this it has been inferred that VCAM-1 expression is subject to redox control.

To investigate whether reactive oxygen metabolites play a role as second messenger molecules in a redox controlled mechanism we investigated whether the expression of VCAM-1 is sensitive to hypoxia. One of the first observations described in chapter 6 was that the TNF-stimulated expression of VCAM-1 is inhibited under hypoxic conditions, while the expression of ICAM-1 is not. This seems to support a role for reactive oxygen metabolites in the intracellular signalling of TNF. It remains to be determined where these species are produced intracellularly. As described in the literature and confirmed in chapter 6, the expression of VCAM-1 (but not ICAM-1) can be inhibited by apocynine , an inhibitor of the phagocytic NADPH-oxidase which produces superoxide anions. This suggests that an enzyme similar to this NADPH-oxidase is a source of superoxide anions in endothelial cells. However, by Western blotting it was not possible to show the expression of essential

components of the NADPH-oxidase (p47-*phox*, p67-*phox*, and gp91*phox*) by endothelial cells. With a more sensitive method other investigators on the other hand have been able to detect p47-*phox*, and p67-*phox* protein expression in endothelial cells. However, since they found no expression of the essential NADPH-oxidase component cytochrome b558, they also conclude that endothelial cells do not express this phagocyte-type enzyme. We further demonstrated that in endothelial cells apocynin inhibits the oxygen-dependent cytochrome P450 monooxygenase. Because other cytochrome P450 inhibitors (cobaltchloride, SKF525a) decreased VCAM-1 (but not ICAM-1) expression, and apocynin inhibited the accumulation of VCAM-1 mRNA, it is likely that the oxygen dependent step in the expression of VCAM-1 is specifically regulated by a cytochrome P450 monooxygenase.

Cytochrome P450 is a potential source of reactive oxygen species, like hydrogen peroxide. Our experiments do not indicate that the basal production of hydrogen peroxide in endothelial cells increases after stimulation with TNF. Moreover, other parameters that reflect the intracellular concentration of reactive oxygen species inside the cell, the pentose phosphate shunt or the membrane lipid peroxidation, do not suggest that TNF increases the intracellular production of hydrogen peroxide. Because it was not feasible to specifically detect the *intra*cellular production of superoxide anions we can not exclude the possibility that the superoxide anion is a second messenger in TNF-stimulated endothelial cells.

Oxygen dependence per se does not necessarily mean that reactive oxygen species are involved in the intracellular signalling of TNF. In chapter 6 we demonstrate that the expression of VCAM-1 (but not ICAM-1) can be decreased by the phospholipase A_2 inhibitor bromophenacylbromide. Cytochrome P450 can metabolize arachidonic acid (AA) liberated by phospholipase A_2 from glycerophospholipids. AA can subsequently be metabolized through the AA ω/ω-1 oxygenase and the AA epoxygenase. Especially the metabolites derived from the AA epoxygenase pathway have been shown to function as second messenger molecules. It is of note here that the activation of the transcription factor IRF-1, which is requisite for the transcriptional induction of VCAM-1, is regulated by phospholipase A_2 activity.

It is well known that the induction of VCAM-1 protein expression is regulated at the transcriptional level. In the present thesis we show for the first time that the expression of VCAM-1 is also regulated *post*-transcriptionally. In chapter 8 we show that the iron chelator desferrioxamine decreases VCAM-1 protein expression, without inhibiting the accumulation of VCAM-1 protein. This effect was specific for VCAM-1, since desferrioxamine did not decrease the stimulated expression of ICAM-1. The incorporation of ^{35}S-methionine into

VCAM-1 protein was decreased by desferrioxamine to the same extent as the expression of VCAM-1 on the endothelial membrane. This strongly suggests a VCAM-1 specific, iron-dependent regulation at the translational level. This translational regulation is not mediated through the low molecular weight iron pool, because after stimulation with TNF the magnitude of this pool does not change. To explain this it is of note that we could demonstrate here that desferrioxamine not only can chelate low molecular weight or free iron but also iron in a protein bound form. Apparently, an iron dependent enzyme is involved in the translational regulation of VCAM-1. The identity of this enzyme is not yet known.

In chapter 7 more evidence is presented for the post-transcriptional (possible translational), specific regulation of VCAM-1. We show that the p38 MAP kinase pathway is functional in endothelial cells, and that its basal activity is stimulated about threefold by TNF. Inhibition of the p38 MAP kinase pathway decreases the expression of VCAM-1, but not the accumulation of VCAM-1 mRNA. The p38 MAP kinase signal transduction pathway is specifically involved in the regulation of VCAM-1, and not of ICAM-1.

VCAM-1 expression is the prelude to a more chronic course of the inflammatory response, and therefore of crucial importance. Our investigations have shown that the expression of VCAM-1 is regulated at different levels, i.e., transcriptionally as well as *post*-transcriptionally. Whether or not these findings can be used to selectively interfere with the accumulation of monocytes (and lymphocytes) into a lesion remains to be demonstrated as yet.

Experimental evidence from animal studies shows that monocytes contribute to the process of restenosis after percutaneous transluminal angioplasty (PTA) through the production of cytokines and growth factors. The patient study described in chapter 9 suggests that the long-term success rate of PTA be determined by the extent to which the patient's monocytes can be activated by lipopolysaccharide (LPS) before the PTA procedure. The higher the *in vitro* production of interleukin-1β (IL-1) before PTA the greater the chance of a patient developing luminal renarrowing. Although, this was determined for the first time in patients this relation was anticipated. Surprisingly, the activation status of circulating granulocytes was inversely proportional with luminal renarrowing at 6 months after PTA. In other words the more active granulocytes in the circulation the greater the lumen of a vessel treated by angioplasty. This is a remarkable finding since granulocytes are well known to aggravate tissue damage after myocardial infarction. The favourable effects of granulocytes in restenosis after PTA need to be confirmed in an independent study. After which the mechanism of how granulocytes mediate this remarkable, and beneficial effect has to be elucidated.

Samenvatting

De ophoping van leukocyten in weefsels wordt gereguleerd in een aantal karakteristieke stappen. In eerste instantie rollen de cellen langs de vaatwand door de vorming van reversibele bindingen tussen gesialyseerde Lewis^X en Lewis^A antigenen op de leukocyt en P- en E-selectines op het endotheel. Omdat de leukocyten zich nu dichtbij de vaatwand bevinden kunnen zij geactiveerd worden door chemotactische factoren en zich vasthechten aan het endotheel. Deze aanhechting wordt gemediëerd door integrine moleculen op de leukocyt en de immunoglobuline-achtige moleculen VCAM-1 en ICAM-1 op het endotheel. Tenslotte migreren de leukocyten naar het onderliggende weefsel in de richting van de bron van een chemotactische factor.

In tegenstelling tot ICAM-1 bevordert VCAM-1 specifiek de ophoping van monocyten, die een belangrijke bijdrage leveren aan chronische ontstekingsreacties.

Recent is gebleken dat VCAM-1 behalve de adhesie van monocyten ook het rollen van deze cellen langs de vaatwand kan bevorderen. Dit betekent dat de adhesie van monocyten aan het endotheel niet afhankelijk is van een gelijktijdige expressie van selectines en VCAM-1. In dit proefschrift staat VCAM-1 centraal.

Onder normale omstandigheden is VCAM-1 niet aantoonbaar op het endotheel. Maar na stimulatie van het endotheel met het inflammatoire cytokine TNF verschijnt VCAM-1 op de endotheliale membraan. De expressie van ICAM-1, welke ook onder normale omstandigheden op het endotheel aanwezig is, wordt door TNF alleen verhoogd. De expressie van VCAM-1 en ICAM-1 houdt zeker 24 tot 48 uur aan. Opvallend is dat de expressie van VCAM-1, in tegenstelling tot die van ICAM-1, te remmen is met verschillende antioxidanten, hetgeen erop duidt dat de signaaltransductie route van VCAM-1 onderhevig is aan redox controle. Om te onderzoeken of reactieve zuurstofmetabolieten deze redox controle mediëren werd eerst onderzocht of de expressie van VCAM-1 gevoelig is voor hypoxie.

Eén van de eerste bevindingen, beschreven in hoofdstuk 6, was dat de TNF gestimuleerde expressie van VCAM-1 is geremd onder hypoxische omstandigheden terwijl de expressie van ICAM-1 gelijk blijft. Dit lijkt een bijdrage van zuurstofradikalen te ondersteunen. De vraag is echter waar deze radikalen worden geproduceerd. Het was reeds bekend dat de expressie van VCAM-1 (en niet die van ICAM-1) kon worden geremd door apocynine, een remmer van het fagocytaire NADPH-oxidase dat superoxide anionen produceert. Dat suggereert dat een dergelijk NADPH-oxidase de bron is van zuurstofradikalen in endotheel. Met behulp van Western blots hebben wij niet kunnen aantonen dat endotheelcellen essentiële componenten

van het NADPH-oxidase (p47-*phox*, p67-*phox*, en gp91*phox*) tot expressie brengen. Hoewel andere onderzoekers het p47-*phox* en het p67-*phox* met een meer gevoelige techniek wel konden aantonen, vonden zij geen expressie van het cytochroom b558 en kwamen dan ook tot de slotsom dat een fagocytair NADPH-oxidase door endotheelcellen niet tot expressie wordt gebracht. Uit ons eigen onderzoek bleek verder dat apocynine in endotheelcellen het zuurstofafhankelijke cytochroom P450 monooxygenase remt. Terwijl andere cytochroom P450 remmers (kobaltchloride, SKF525a) ook selectief de expressie van VCAM-1 (en niet van ICAM-1) remmen. Omdat apocynine ook de ophoping van het VCAM-1 mRNA remt lijkt het waarschijnlijk dat de zuurstofafhankelijke stap in de transcriptionele regulatie van VCAM-1 door een cytochroom P450 monooxygenase wordt gekatalyseerd.

Cytochrome P450 monooxygenase is een potentiële bron van reactieve zuurstofmetabolieten, zoals waterstofperoxide. Uit onze experimenten is niet gebleken dat de basale productie van waterstofperoxide door endotheelcellen toeneemt na stimulatie met TNF. Ook andere indicatoren voor een verhoging van de intracellulaire waterstofperoxide concentratie (pentose fosfaat shunt activiteit, membraangebonden lipide peroxidatie) duiden er niet op dat endotheel na stimulatie met TNF meer waterstofperoxide produceert. Omdat er gedurende dit onderzoek geen geschikte manier voor handen was om de *intra* cellulaire concentratie van superoxide anion te meten hebben we hier niet kunnen uitsluiten dat dit radikaal een rol vervult als intracellulaire second messenger.

Het feit dat de VCAM-1 expressie zuurstofafhankelijk is hoeft echter niet perse te betekenen dat reactieve zuurstofmetabolieten zijn betrokken bij de intracellulaire signaaloverdracht. In hoofdstuk 6 wordt beschreven dat de expressie van VCAM-1 kan worden geremd door de fosfolipase-A_2 remmer bromophenacylbromide. Het cytochroom P450 arachidonzuur (AA) kan metaboliseren dat door fosfolipase-A_2 wordt vrijgemaakt uit glycerofosfolipiden. AA kan vervolgens worden gemetaboliseerd door het AA ω/ω-1 oxygenase en het AA epoxygenase. Vooral produkten van de AA-epoxygenase route kunnen functioneren als second messengers. Of deze enzymen ook een specifieke bijdrage aan de transcriptionele regulatie van VCAM-1 leveren moet nog worden onderzocht. Het is hierbij van belang te vermelden dat de activering van de transcriptie factor IRF-1, welke de expressie van VCAM-1 mede bepaalt, afhankelijk is van fosfolipase-A_2 activiteit.

Zoals reeds bekend en opnieuw door ons onderzoek wordt bevestigd vindt de regulatie van VCAM-1 expressie plaats op het niveau van de transcriptie. Zoals beschreven in hoofdstuk 8 bleek tot onze verrassing dat de ijzerchelator desferrioxamine de expressie van het VCAM-1 eiwit op de endotheliale celmembraan remt, zonder de ophoping van VCAM-1 mRNA

negatief te beïnvloeden. Dit effect was specifiek voor VCAM-1 aangezien de door TNF gestimuleerde expressie van ICAM-1 niet door desferrioxamine werd geremd. Omdat de inbouw van ^{35}S-methionine in het VCAM-1 eiwit in gelijke mate werd geremd als de membraan expressie van VCAM-1, bestaat er kennelijk ook een VCAM-1 specifiek, ijzer afhankelijke regulatie op het niveau van de translatie. Deze translationele regulatie is niet afhankelijk van de laag moleculaire (vrije) ijzer pool want na stimulatie met TNF veranderde de grootte van de vrije ijzer pool niet. Om het effect van desferrioxamine te kunnen begrijpen is het van belang te weten dat desferrioxamine niet alleen in staat vrij ijzer maar ook enzymgebonden ijzer te cheleren. Blijkbaar speelt een ijzer afhankelijk enzym een rol in de specifieke translationele regulatie van VCAM-1. De identiteit van dit ijzer afhankelijke enzym is nog onbekend.

In hoofdstuk 7 staan meer aanwijzingen beschreven voor een post-transcriptionele (mogelijk translationele) en specifieke regulatie van VCAM-1. Wij hebben aangetoond dat de p38 MAP kinase route functioneert in endotheelcellen en dat de basale activiteit ervan door TNF ongeveer tot het drievoudige wordt gestimuleerd. Wanneer het p38 MAP kinase wordt geremd neemt de expressie van het VCAM-1 eiwit af, maar niet de ophoping van het VCAM-1 mRNA. Remming van het p38 MAP kinase heeft geen invloed op de expressie van het ICAM-1 eiwit, in dat opzicht is deze signaaltransductie route dus specifiek betrokken bij de expressie van VCAM-1.

Gedurende een ontstekingsreactie vormt de expressie van VCAM-1 in feite de opmaat naar een meer chronisch verloop van de reactie. Uit ons onderzoek is gebleken dat de expressie van VCAM-1 op meerdere niveaus wordt gereguleerd. Of daarmee aanknopingpunten kunnen worden gevonden om selectief de ophoping van monocyten (en lymfocyten) in een lesie te voorkomen zal nog moeten blijken.

Uit dierexperimentele studies is reeds bekend dat monocyten kunnen bijdragen aan de ontwikkeling van restenose na percutane transluminale angioplastie (ofwel de Dotterbehandeling) door de productie van cytokinen en groeifactoren. Uit het patiëntenonderzoek, beschreven in hoofdstuk 9, is gebleken dat het succes van een Dotterbehandeling op de lange termijn wordt bepaald door de mate waarin de monocyten van een patiënt, voorafgaand aan de Dotterbehandeling, na een activatieprikkel (lipopolysaccharide) *in vitro* IL-1β produceren. Hoe sterker daarbij de activering des te slechter de prognose. Alhoewel hier voor het eerst in patiënten vastgesteld, is rol van monocyten in het opnieuw vernauwen van de kransslagader na een geslaagde Dotterbehandeling in de lijn der verwachting. Tot onze verrassing bleek echter de mate van

activatie van granulocyten juist omgekeerd evenredig met het optreden van restenose na een geslaagde Dotterbehandeling. Met andere woorden hoe actiever de granulocyt des te groter het lumen van het behandelde vat op de lange termijn. Dit is opmerkelijk omdat van granulocyten bekend is dat zij de schade aan het hartweefsel na een infarct juist vergroten. Vanzelfsprekend zullen de resultaten van dit onderzoek eerst in een onafhankelijke studie moeten worden bevestigd, en zal vervolgens moeten worden onderzocht hoe granulocyten deze opmerkelijke, gunstige rol vervullen.

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Curriculum Vitae

Anna Pietersma[1)] werd geboren te Sneek op 13 oktober 1965. In 1984 behaalde zij het Atheneum-B diploma aan het Carolus Clusius College te Zwolle. Daarna begon zij haar studie aan de Landbouwuniversiteit Wageningen.

Daar specialiseerde ze zich in de biochemische en fysiologische aspecten van de voeding van de mens. Na de hoofdvakken Voedingsleer (begeleid door prof. C.E. West van de vakgroep Humane Voeding) en Toxicologie (begeleid door dr. J.H.M. Temmink van de vakgroep Toxicologie) vertrok zij naar de Verenigde Staten om deel te nemen aan een expeditie naar de Mount Everest. In het kader van haar praktijktijd verrichtte zij daar onderzoek naar de effecten van extreme hoogte en koude op het energiemetabolisme en de lichaamssamenstelling van bergbeklimmers dit onder leiding van prof. R.D. Reynolds (USDA/ Beltsville Human Nutrition Research Center). Terug op Nederlandse bodem rondde zij haar studie af met een hoofdvak Biochemie, daarin werkte zij onder leiding van dr. W. van Berkel van de vakgroep Biochemie aan de zuivering en karakterisering van een nieuw FAD-afhankelijk styreen monooxygenase. Na haar afstuderen in 1990 trad zij in dienst van het instituut Biochemie van de Erasmus Universiteit Rotterdam.

Onder leiding van dr. W. Sluiter en prof. J.F. Koster verrichtte zij daar van 1990 tot 1993 voor de Nederlandse Hartstichting onderzoek naar de interactie tussen endotheelcellen en granulocyten tijdens hypoxie en reoxygenatie, en leverde haar bijdrage aan een klinische studie naar de rol van granulocyten en monocyten in de ontwikkeling van restenose na percutane transluminale angioplastie van een coronair vat, beter bekend als de Dotterbehandeling. In 1994 begon zij aan het onderzoek naar de mogelijke rol van reactieve zuurstofmetabolieten als intracellulaire signaalstoffen in endotheelcellen (NHS 93.124). De resultaten hiervan staan beschreven in dit proefschrift.

Vanaf februari 1999 werkt zij voor de afdeling 'Regulatory Affairs' van Organon NV te Oss.

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Publications

1. Pietersma A, de Jong N, Sluiter W, and Koster JF. Studies on the interaction of leukocytes and the myocardial vasculature. I Effect of hypoxia on the adherence of granulocytes. **Mol Cell Biochem 1992;116:197-202.**

2. Sluiter W, Pietersma A, Lamers JMJ, Koster JF. Leukocyte adhesion molecules on the vascular endothelium. Their role in the pathogenesis of cardiovascular disease and the mechanisms underlying their expression. **J Cardiovascular Pharmacol 1993;22(Suppl.4):S37-S44.**

3. Pietersma A, de Jong N, Koster JF, Sluiter W. Extreme hypoxia decreases the adherence of granulocytes to endothelial cells *in vitro*. **Ann N Y Acad Sc 1994;723,486-487.**

4. Pietersma A, de Jong N, Koster JF, Sluiter W. Effect of hypoxia on the adherence of granulocytes to endothelial cells *in vitro*. **Am J Physiol 1994;267:H874-H879.**

5. Pietersma A, Kofflard M, van de Merbel-de Wit LEA, Stijnen Th, Koster JF, Serruys PW, Sluiter W. Late lumen loss after coronary angioplasty is associated with the activation status of circulating phagocytes before treatment. **Circulation 1995;91:1320-1325.**

6. Pietersma A, de Jong N, Koster JF, Sluiter W. Hypoxia and endothelial cell adhesiveness. **Proceedings of the NATO/FEBS course on Molecular Mechanisms of Transcellular Signalling: From the Membrane to the Gene. 1995.**

7. De Maat M, Pietersma A, Kofflard M, Sluiter W, Kluft C. Coronary artery disease and inflammation: association of coronary artery disease with fibrinogen, C-reactive protein, cytokines and smoking. **Artherosclerosis, 1996;121:185-191.**

8. Sluiter W, de Vree WJA, Pietersma A, Koster JF. Prevention of late lumen loss after coronary angioplasty by photodynamic therapy: Role of activated neutrophils. **Mol Cell Biochem 1996;157:233-238.**

9. Pietersma A, Tilly BC, Gaestel M, de Jong N, Lee JC, Koster JF, Sluiter W. P38 Mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. **Biochem Biophys Res Commun. 1997;230:44-48.**

10. Pietersma A., N. de Jong, L.E.A. de Wit, R.G. Kraak-Slee, J.F. Koster, W. Sluiter. Evidence against the involvement of multiple radical generating sites in the expression of the vascular cell adhesion molecule-1. **Free Radical Research. 1998;28:137-15.**

11. Reynolds R. D., Lickteig J. A., Deuster P. A., Howard M. P, Seale J. L., Conway J. M., Rumpler W.V., Pietersma A, de Stoppelaar J. S. Deurenberg P. Energy metabolism increases, regional body fat decreases, and muscle mass is maintained during a climb up Mt. Everest. **Submitted.**

12. Pietersma A., R. Kraak-Slee, N. de Jong, L.E.A. de Wit, H.A.A. van Heugten, J.F. Koster, W. Sluiter. The role of iron in the expression of VCAM-1. **Submitted.**