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Co-expression of ICAM-1, VCAM-1, ELAM-1 and Hsp60 in human arterial and venous endothelial cells in response to cytokines and oxidized low-density lipoproteins

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Abstract T-cells and monocytes are the first cells infiltrating the arterial intima during the early stages of atherogenesis. Recently our laboratory has provided evidence that T-cells isolated from atherosclerotic intima reacts against heat shock protein 60 (Hsp60). Transmigration of activated T-cells into the intima is mediated by adhesion molecules (ICAM-1; VCAM-1; ELAM-1) expressed on activated endothelial cells. Here we studied the potential of cytokines (TNF-α, IFN-γ, IL-1), *Escherichia coli* lipopolysaccharide (LPS), native and oxidized low-density lipoprotein (LDL; oxLDL) and high temperature to induce adhesion molecules as well as Hsp60 and Hsp70 expression in human endothelial cells (EC). On Northern blots, a strong signal for ICAM-1, VCAM-1 and ELAM-1 was detected after 4 h, which thereafter declined, but did not reach the basal level of untreated control cells. Heat shock induced the expression of Hsp60 and Hsp70 but not of adhesion molecules. EC were cultivated in serum-free medium, which led to the expression of adhesion molecule transcripts. Addition of LDL or oxLDL to these ECs did not alter the expression of these transcripts. The production of adhesion molecule proteins was analysed by flow cytometry. In human venous endothelial cells (HVEC) and human arterial endothelial cells (HAEC) ICAM-1 and VCAM-1 production was permanently highly induced, whereas the high level of ELAM-1 production at 4 h disappeared after 24 h. Furthermore, only HAEC, but not HVEC, produced ICAM-1, VCAM-1 and ELAM-1 after stress by moderately and highly oxLDL. LDL and oxLDL did not induce the production of Hsp60 and Hsp70. The present study demonstrates the co-expression of Hsp60 and adhesion molecules in arterial and venous EC in response to cytokine and LPS exposure, and that oxLDL is an efficient inducer of adhesion molecules in arterial EC and not in venous EC. These features provide the prerequisites for a cellular immune reaction against Hsp60 expressed by stressed EC in the inital stages of atherosclerosis.

INTRODUCTION

The infiltration of circulating monocytes and T-lymphocytes into the arterial intima is the earliest detectable event in the development of atherosclerosis (Hansson et al 1988; Xu et al 1990). The high proportion of these intima-infiltrating T-lymphocytes that express major histocompatibility complex class II (HLA-DR) and interleukin-2 receptors indicate an activated state (Hansson et al 1989; Stemme et al 1992). It is also well established that endothelial cell adhesion molecules play an important role in the emigration of T-lymphocytes and monocytes from the blood into foci of inflammation (Collins et al 1993; Springer 1994), and growing evidence suggests that a

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similar process occurs in atherosclerosis. For instance, intercellular adhesion molecule-1 (ICAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1) are strongly expressed by intimal cells in human atherosclerotic lesions (Poston et al 1992; Printseva et al 1992; Van der Wal et al 1992). In rabbits, the vascular cell adhesion molecule-1 (VCAM-1) is also upregulated in endothelium overlaying atherosclerotic plaques (Li et al 1993). The expression of adhesion molecules in endothelial cells (EC) can be induced by various stimuli, including cytokines and other inflammatory mediators of exogenous origin, or produced by cells in the arterial intima (Ross 1993). We have previously shown that ICAM-1 is expressed in the endothelium of rat aorta after injection of bacterial endotoxin, and that this expression is related to an increased adhesion of monocytes and T-lymphocytes to the aortic endothelium (Seitz et al 1996).

In addition to adhesion molecules, specific antigens expressed in the arterial intima may be crucial to lymphocyte recruitment. Xu and co-workers found that immunization with mycobacterial heat shock protein 65 (Hsp65) can induce atherosclerotic lesions in normocholesterolemic rabbits and that a population of T-lymphocytes isolated from these lesions specifically responds to Hsp65 (Xu et al 1992, 1993). Mycobacterial Hsp65 belongs to a family of proteins of high sequence homology including the mammalian homologue Hsp60, which acts as a chaperone and protects cells against various forms of stress (Ellis and van der Vies 1991). The high degree of homology between mycobacterial Hsp65 and mammalian Hsp60 suggests that Hsp65 reactive T-lymphocytes can react against Hsp60 expressed by intimal cells in stressed arteries. These observations suggested that simultaneous expression of adhesion molecules and Hsp60 at sites predisposed to atherosclerosis is prerequisite for the interaction of Hsp65/60 reactive T-cells and EC, and the subsequent transmigration of these T-cells into the intima (Wick et al 1992, 1995). Thus far, most studies on Hsp expression in vitro have been carried out in cell-lines (Fincato et al 1991; Ferm et al 1992), but little is known about the regulation of Hsp60 expression in primary cultures of human arterial and venous EC.

It is well established that oxidized low-density lipoprotein (oxLDL) is a main risk factor for atherogenesis (Witztum 1994). OxLDL was found in atherosclerotic lesions in vivo (Jürgens et al 1993), and can induce monocyte chemotactic protein-1 in EC and smooth muscle cells in vitro (Cushing et al 1990). However, the direct effect of oxLDL on adhesion molecule expression in human arterial and venous EC of adult individuals is still unknown. A comparative study of adhesion molecule expression by arterial and venous EC was deemed necessary,since vascular endothelium from different anatomical compartments possess different properties in vivo (Page et al 1992). Thus, the expression of VCAM-1 cannot be detected in aortic and pulmonary arterial endothelial cells from healthy human donors, whereas VCAM-1 is expressed in ECs of coronary arteries (Page et al 1992). Differential responses of venous and arterial EC of large vessels to certain risk factors, in addition to a higher blood pressure could be a possible explanation why only arterial vessels undergo atherogenesis. To test this hypothesis, we analyzed the response of venous and arterial EC grown under the same stress conditions. Here, we show that the stimuli leading to Hsp60 expression also induces adhesion molecule expression in human arterial and venous EC. We further demonstrate that chronic exposure of moderately- and highly-oxidized LDL to EC induce ICAM-1, VCAM-1 and ELAM-1 expression only in arterial, and not in venous, EC. This suggests that HAEC and HVEC represent different EC phenotypes and, therefore, exhibit different responses to oxLDL. Our findings provide new insights into the role of EC in the immunologically mediated induction of atherosclerosis.

METHODS

Endothelial cell preparation and culture

The Department of Vascular Surgery, University of Innsbruck, Austria provided the human cells: HVEC were isolated from saphenous veins obtained from patients undergoing bypass operations and HAEC were isolated from femoral arteries obtained as organ donations from young people killed in traffic accidents. The vascular lumen was flushed with cold RPMI 1640 (Seromed, Vienna), filled with collagenase IV (1 mg/ml, Sigma, Munich) in RPMI 1640 and incubated at room temperature for 20 min. EC were harvested by rinsing the lumen with cold RPMI 1640, centrifuging at 300 *g* for 5 min and plating on 0.2 % gelatin (2% solution, Type B, Sigma) coated culture flasks. EC were cultivated in culture medium (M199 and RPMI 1640, 1:1) supplemented with 20% fetal calf serum (FCS, Seromed), endothelial cell growth supplement (ECGS, 100 µg/ml, Sigma) and preservative-free heparin (100 µg/ml, Sigma). Five different HVEC lines (male donors 72 and 72 years old, respectively; female donors 62, 65 and 72 years old, respectively) and three HAEC lines (male donors 25 and 26 years old, respectively; and one female donor 26 years old) were used in this study. Donors for HVEC differ from the healthy donors for HAEC because they received therapeutic medication. We could not exclude the influence of this medication on EC behavior in our experiments, therefore, we performed the experiments also with HUVEC. Cells were identified as EC by immunofluorescence staining analysis of factor VIII antigen production and

during the experiments cells were identified as EC by ELAM-1 production after TNF-α treatment, which was described to be a specific EC marker (Gearing and Newman, 1994). The third to fifth passage of EC was used for all experiments, because after passage number 7 EC stopped proliferating, dedifferentiated (no ELAM-1 expression after TNF-α treatment), became elongated and subsequently died. Each EC-line was treated with different stressors and adhesion molecules and Hsp-expression was analyzed on Northern blot and FACS analysis or immunoblot.

Application of stress to endothelial cells

EC were passaged on culture dishes (35 × 10 mm, Falcon, Plymouth, UK) pre-coated with 0.2 % gelatin at 37°C for 30 min and cultivated until they reached confluence. The cells were then washed with phosphate-buffered saline (PBS, pH 7.2) and cultivated in new medium (M199/RPMI 1640) supplemented with various stressors (TNF-α 10 ng/ml; Boehringer/Mannheim), IL-1 10 ng/ml; Boehringer/Mannheim); *E. coli* lipopolysaccharide (LPS, 3 μg/ml, Sigma); IFN-γ (30 ng/ml), native LDL and oxidized LDL (80 μg/ml), or EC were exposed to elevated temperature (42°C, 30 min). For experiments with LDL and oxLDL, ECs were cultivated in a serum-free medium for 24 h. Thereafter, the medium was removed and new serum-free medium supplemented with LDL or oxLDL was added. The medium was removed at different time periods and cells were washed and collected for Northern blots, FACS analyses or immunoblots. EC viability during cultivation in the serum-free medium was analyzed by the measuring of apoptosis by propidium iodide staining of EC-nuclei followed by flow cytometry (Nicoletti et al 1991). Apoptose was not significantly increased in EC cultivated in serum-free medium compared to EC cultivated in serum-supplemented medium.

Preparation and characterization of oxLDL

Low-density lipoprotein (LDL) was isolated from EDTA-plasma of three normolipemic, fasting (12–14 h), young male donors (<30 years), as described previously (Jürgens et al 1990). The subjects had low Lp(a) levels (<5 mg/dl). LDL was isolated by differential ultracentrifugation within a density range of 1.020–1.050 g/ml. After dialyzing LDL against 0.01 M PBS, LDL oxidation (1.5 mg/ml total lipoprotein) was performed at 37°C in the presence of 10 μM $CuCl_2$. After 1, 4 and 24 h, respectively, aliquots were withdrawn and the copper-mediated oxidation was stopped by an addition of 1 mg/ml EDTA. The samples were dialyzed against 0.1 M Tris-HCl, pH 7.4 containing 1 g/l EDTA. Lipid hydroperoxides were estimated according to a previously developed method (El-Saadani et al 1989). Change in the LDL surface charge was assessed by estimating electrophoretic mobility on agarose gels (Jürgens et al 1990). The formation of newly generated epitopes on apolipoprotein B (apo B) was recorded with poly- and monoclonal antibodies to modified apo B (Jürgens et al 1986, 1990, 1992; Hammer et al 1995) by means of a solid phase dissociation-enhanced lanthanide fluorescence immunoassay, as described earlier (Hammer et al 1995; Jürgens et al 1992). Anti-apo B was a rabbit polyclonal antibody purchased from Behringwerke (Marburg/Lahn, Germany). Anti-4-HNE-apo B LDL, modified with 4-hydroxynonenal (4-HNE) (Jürgens et al 1986, 1990), a major breakdown product of lipid hydroperoxides, was used to prepare a polyclonal antibody in rabbits. LDL modified with malondialdehyde (MDA), another major breakdown product of lipid hydroperoxides (Hammer et al 1995), was used for preparation of anti-MDA-apo B, a polyclonal antibody raised in rabbits. Both antibodies also reacted with oxidized, but not with native LDL. Anti-ox-apo B (OB04) was a monoclonal antibody raised against copper-oxidized LDL and characterized to react specifically with oxidized apo B containing lipoproteins (Hammer et al 1995).

RNA isolation and Northern blots

Total RNA was isolated from cells according to a standard protocol (Chomczynski and Sacchi 1987). Ten μg RNA per lane were electrophoresed in a 1% formaldehyde agarose gel and blotted on a Nylon membrane (Zeta probe, BioRad, Richmond, VA) by capillary transfer. The RNA on filters was UV-crosslinked and hybridized with ^{32}P labeled cDNA (^{32}P dCTP from Amersham, London, and random primed labeling kit from Boehringer/Mannheim) for ICAM-1, VCAM-1, ELAM-1, Hsp60, Hsp70, GAPDH at 63°C in hybridization-buffer (Church and Gilbert 1984). After three washing steps, filters were exposed to X-OMAT X-ray films (Kodak, Rochester, NY) at –70°C.

The following cDNA clones were used: pSJ 60 for Hsp60 (BamH1-Nde 1 fragment; Jindal et al 1989); pSJ 70 for Hsp70 (BamH1-Nde 1 fragment); ELAM-1 cDNA in pCDM 8 vector (Xba I fragment; Hession et al 1990); VCAM-1 cDNA in pBS vector (EcoR1-Hind III fragment; Cybulsky et al 1991) ; ICAM-1 cDNA in pBS vector (Xho I fragment; Voraberger et al 1991; gift from Dr Vogetseder, Innsbruck); Glyceraldehyde-3-phosphate dehydrogenase cDNA (GAPDH, Dugaiczyk et al 1983)

FACS analysis

For flow cytometric analysis, EC were removed from culture dishes with Versene (BioWhittaker, Wakersville, MD) and incubated in PBS containing 1% bovine serum

albumin (BSA, Sigma) with appropriately diluted monoclonal antibodies at 4°C for 30 min. After three washes, the cells were incubated with a FITC-conjugated rabbit anti-mouse Ig (An der Grub, Austria) at 4°C for 30 min, and washed again. FACS analysis were performed on a FACScan (Becton Dickinson, Plymouth, UK). Expression of adhesion molecules on HAEC and HVEC was successively performed with the same instrument settings.

The following primary antibodies were used: anti ICAM-1 Phycoerythrin conjugated (PharMingen, San Diego, CA) diluted 1:30; anti VCAM-1 (clone 1G11B1, Monosan, Netherlands) diluted 1:30; anti ELAM-1 (clone ENA 1; Monosan), diluted 1:25.

Immunoblot

EC were harvested in extraction buffer (50 mM Tris-HCl, pH 7.4; 0.154 mM NaCl; 1 mM phenylmethylsulfonylfluoride (PMSF); 1 mM EDTA; 0.5 % Triton X-100, 0.05 % sodium dodecyl sulfate, SDS) and protein content was measured with a BioRad protein assay kit (BioRad). Samples were diluted 1:2 (vol/vol) in electrophoresis sample buffer (5% β-mercaptoethanol, 15% glycerol; 3% SDS, 100 mM tris(hydroxymethyl)aminomethane, pH 6.8) and resolved on a 9% polyacrylamide gel under reducing conditions. Proteins were blotted onto nitrocellulose (BA 85, Schleicher and Schuell) in 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3 at 75 mA for 20 h. After blocking with 2% milk powder (Sigma) in PBS for 1 h, the blots were probed with an anti-human Hsp60 antibody (clone II-13, diluted 1:10 000), or an anti-human Hsp70 antibody (SPA-810, StressGene) diluted 1:500 in 1% BSA/PBS for 1 h. Antibody binding was visualized by horseradish peroxidase conjugated rabbit anti-mouse Ig (Dakopatts, diluted 1:500) for 1 h, followed by incubation in 4-chloro-1-naphthol/hydrogen peroxide (Sigma) in 50 mM Tris-HCl. Densitometric analysis of immunoblots was performed with the Scan Pack II System (Biometra, Göttingen; Germany).

Statistics

Students paired *t*-test were used for statistical evaluation.

RESULTS

Expression of adhesion molecules in human arterial and venous endothelial cells after cytokine or endotoxin treatment

Expression of mRNA

Confluent monolayers of EC from venous and arterial vessels were stressed with TNF-α, IL-1, LPS or IFN-γ. The mRNA expression of ICAM-1, VCAM-1 and ELAM-1 was analyzed by hybridization of Northern blots of total RNA with specific radiolabeled cDNA probes. The experiments revealed a similar expression pattern of these molecules by HAEC and HVEC. A strong hybridization signal for ELAM-1 and VCAM-1 and a weaker signal for ICAM-1 were detected after 4 h. Thereafter, mRNA levels decreased at 12 and 24 h, but did not reach basal levels of untreated EC (Fig. 1). All stressors were equally effective in inducing adhesion molecule mRNA expression (Fig. 1). The time course of adhesion molecule mRNA expression exhibited no difference between venous and arterial endothelial cells after cytokine or endotoxin treatment.

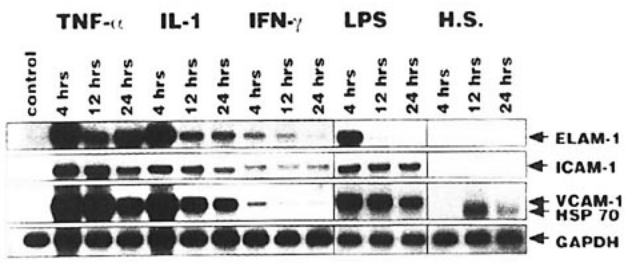

Fig. 1 Northern blot analysis of heat shock protein and adhesion molecule expression. Confluent EC monolayers were treated with TNF-α (10 ng/ml), IL-1 (10 ng/ml), IFN-γ (30 ng/ml), *E. coli* lipopolysaccharide (LPS, 3 μg/ml) or heat shock (42°C for 30 min), respectively and expression of ICAM-1, VCAM-1, ELAM-1, Hsp70 and GAPDH was analysed after 4, 12 and 24 h. The figure shows the results from HVEC (72 year old male donor), and is also representative for the other HVEC-HAEC-lines.

Protein expression

Maximal expression of ELAM-1 occurred on the cell surface of both HAEC and HVEC after 4 h of stress and rapidly declined to basal levels despite the continuous presence of cytokines and LPS (Fig. 2E,F). VCAM-1 was continuously upregulated up to 12 h. In HVEC, VCAM-1 subsequently declined to lower levels in the presence of cytokines and endotoxin (Fig. 2C). In HAEC, VCAM-1 expression was further induced at 24 h in the presence of TNF-α and LPS (Fig. 2D), whereas in presence of IL-1 VCAM-1 expression at 24 h remained equal to that at 12 h (Fig. 2C). ICAM-1 was basally expressed on all tested EC and increased continuously from 4 to 24 h on HAEC and HVEC in the presence of TNF-α, IL-1, LPS and also IFN-γ (Fig. 2A,B), as described by others for HUVECs (Bevilacqua 1993). Even a weak increase of mRNA expression of all three adhesion molecules was detected after IFN-γ treatment, this pattern was not reflected on the protein level. A minor amount of ICAM-1 could be induced on HVEC and HAEC by this cytokine (Fig. 2A,B), whereas VCAM-1 and ELAM-1 expression was not

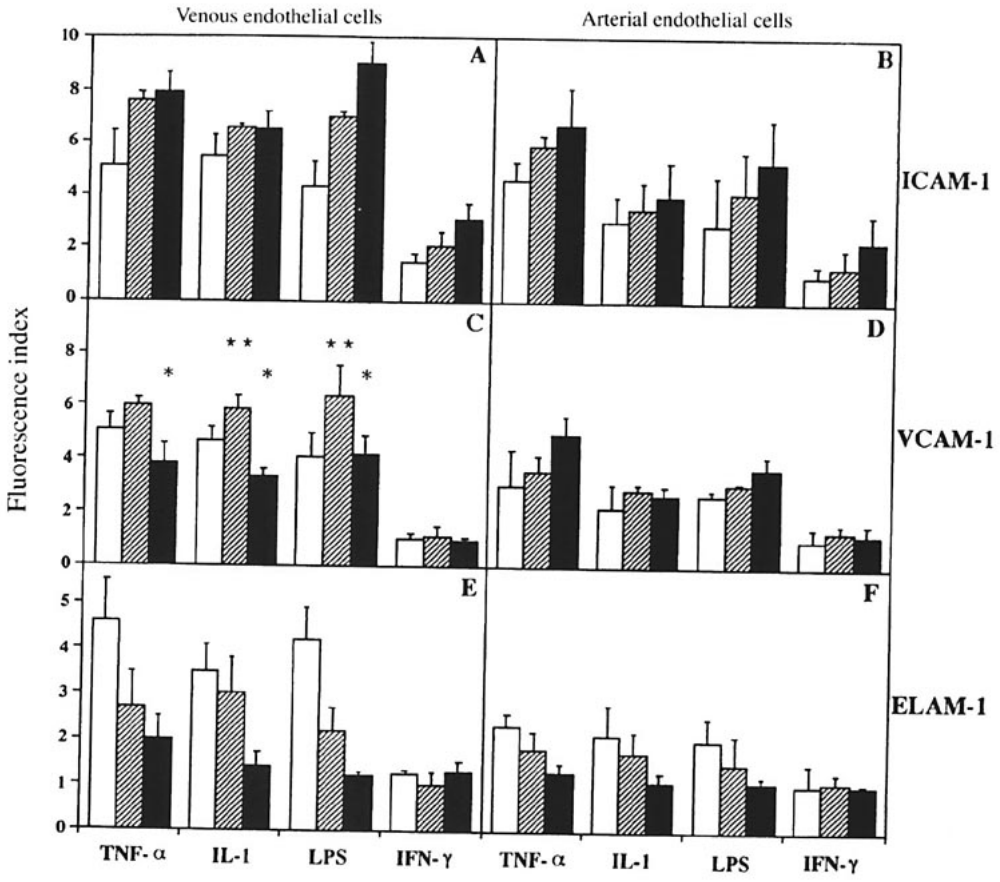

Fig. 2 FACS analysis of adhesion molecule expression on venous and arterial EC. Cells were treated as described in Fig. 1. The fluorescence index was calculated as the ratio of the mean fluorescence intensity of stressed EC to unstressed EC. Mean values and standard deviations of 5 independent experiments are shown. □ 4 h; ▨ 12 h; ■ 24 h. *Significant difference from stressed EC at 12 h, $P < 0.05$. **No significant difference from stressed EC at 4 h.

induced (Fig. 2C–F). Induction of adhesion molecule expression was stronger in HVEC than in HAEC, therefore we tested the production of adhesion molecules in HUVEC by these different stressors. In HUVEC induction of adhesion molecule expression was as equally high as in HVEC (not shown). These findings suggest that EC from venous origins produce more ICAM-1, VCAM-1 and ELAM-1 than HAEC after stimulation by cytokines and LPS.

Heat shock (42°C for 30 min) of EC had no effect on adhesion molecule expression on the levels of RNA (Fig. 1) and protein accumulation (results not shown).

Characteristics of oxidized LDL

The biological properties of oxLDL may vary among different donors or preparations. Therefore, we used three different LDL preparations and tested their susceptibility to oxidation by $CuCl_2$. As shown in Table 1, freshly isolated LDL samples contained no lipid hydroperoxides (LPO). During copper-induced oxidation all three LDL preparations showed similar changes of LPO content [Table (A)], that is, decreased after long oxidation periods (24 h), because LPO were further degraded. The oxidative modification of apo B continued up to 24 h, as evident from increased electrophoretic mobility. Decreased native apo B-epitopes and increased newly generated epitopes during oxidation showed differences between the three LDL preparations at different stages of oxidation [Table (B)]. LDL from donor 2 revealed a slower formation of new epitopes, although a faster decrease of native apo B epitopes compared to LDL preparations from donors 1 and 3.

To standardize experiments, EC were cultivated in a serum-free medium to rule out any effect of LDL or oxidized LDL content in fetal calf serum. In pilot experiments, different concentrations of oxLDL (20, 40, 60, 80 and 160 μg/ml) were tested in our EC-culture system. Induction of adhesion molecule expression in arterial EC

Table Characterization of oxidized LDL

(A) Increase in content of lipid hydroperoxides and electrophoretic mobility during LDL oxidation

		LPO (nmol/mg LDL)			EM (mm)		
t_{ox}	**Donor**	**1**	**2**	**3**	**1**	**2**	**3**
0 h		0.9	0	0	12	10	12
1 h		8.1	0	0	14	12	14
4 h		64.2	57.0	73.5	25	17	20
24 h		36.3	27.9	26.4	41	40	46

(B) Decrease of native apo-lipoprotein B epitopes and increase in newly generated epitopes during LDL oxidation

		Anti-apo B (%)			Anti-4-HNE-apo B (%)			Anti-MDA-apo B (%)			Anti-ox-apo B (%)		
t_{ox}	**Donor**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
0 h		100	100	100	0	0	0	0	0	0	0	0	0
1 h		104	107	113	4	5	0	13	4	28	7	3	6
4 h		65	26	59	36	10	7	47	29	31	28	7	29
24 h		0	0	0	100	100	100	100	100	100	100	100	100

Low-density lipoprotein (LDL) was isolated from plasma of three normolipemic young (<30 a) male donors and oxidized by 10 μM $CuCl_2$. After 1, 4 and 24 h of incubation (t_{ox}) aliquots were withdrawn and the degree of oxidation was determined. Lipid hydroperoxide (LPO) content in LDL particles and their electrophoretic mobility (EM) are shown in (A). Specific binding of antibodies to native apo B at 0 h and to modified LDL at 24 h was set to 100%, respectively. The antibody reaction to other LDL samples was related to these values (B).

could only be detected at concentrations of 80 μg/ml and 160 μg/ml medium reflecting average in vivo concentrations in humans (results not shown).

Expression of adhesion molecules after native LDL and oxidized LDL treatment

Expression of mRNA

Venous and arterial endothelial cell monolayers were exposed to native-, minimally-, moderately- and highly-oxidized LDL (80 μg/ml) for 12, 24 and 48 h, respectively. Weak expression of ICAM-1, VCAM-1 and ELAM-1 mRNA was detected in untreated HVEC and HAEC cultivated in serum-free medium for 24 h. This mRNA expression did not change during further cultivation in serum-free medium and was not further induced by an addition of native or oxidized LDL (results not shown).

Protein expression

HVEC and HAEC cultivated in serum-free medium showed no VCAM-1 and ELAM-1 expression, but basal expression of ICAM-1 was seen as determined by FACS (not shown). Surprisingly, expression of cell adhesion molecules was only detected in HAEC, not HVEC or HUVEC (not shown), and only after addition of moderately- or highly-oxidized LDL. As shown in Figure 3A, moderately- and highly-oxidized LDL induced the expression of all three adhesion molecules, which increased continuously for up to 48 h. In contrast to TNF-α, IL-1 and LPS mediated stress, oxLDL led to a continuous increase of ELAM-1 expression (Fig. 3A; f). The extent of ICAM-1, VCAM-1 and ELAM-1 expression was highest when moderate-oxLDL was used as stressor. Figure 3B shows a representative histogram of ELAM-1 expression after oxLDL(4) exposure for 48 h.

Independent of the LDL fractions from the three donors used in this experiments, adhesion molecule expression was only induced in arterial EC. The three LDL preparations showed no significant difference in their ability to induce adhesion molecules.

Expression of Hsp60 and Hsp70 in human venous and arterial endothelial cells

Expression of mRNA

Venous and arterial EC were analysed for Hsp60 mRNA expression under normal and stressed culture conditions. Signals of Hsp60 mRNA was detected in all venous and arterial EC cultures under normal conditions (control in Fig. 4A). Expression of Hsp60 mRNA was further induced by high temperature, incubation with TNF-α (Fig. 4A) and IL-1, IFN-γ, endotoxin but not by LDL and oxLDL (not shown). In contrast, Hsp70 mRNA expression was not present in untreated cells, but was induced after heat treatment and reached a maximal signal at 12 h, and thereafter decreased to nearly basal levels (Fig. 1).

Protein expression

Hsp60 is basally expressed in EC and could be further induced after stress by TNF-α high temperatures, as shown in Figure 4B, and IL-1, IFN-γ and endotoxin, but

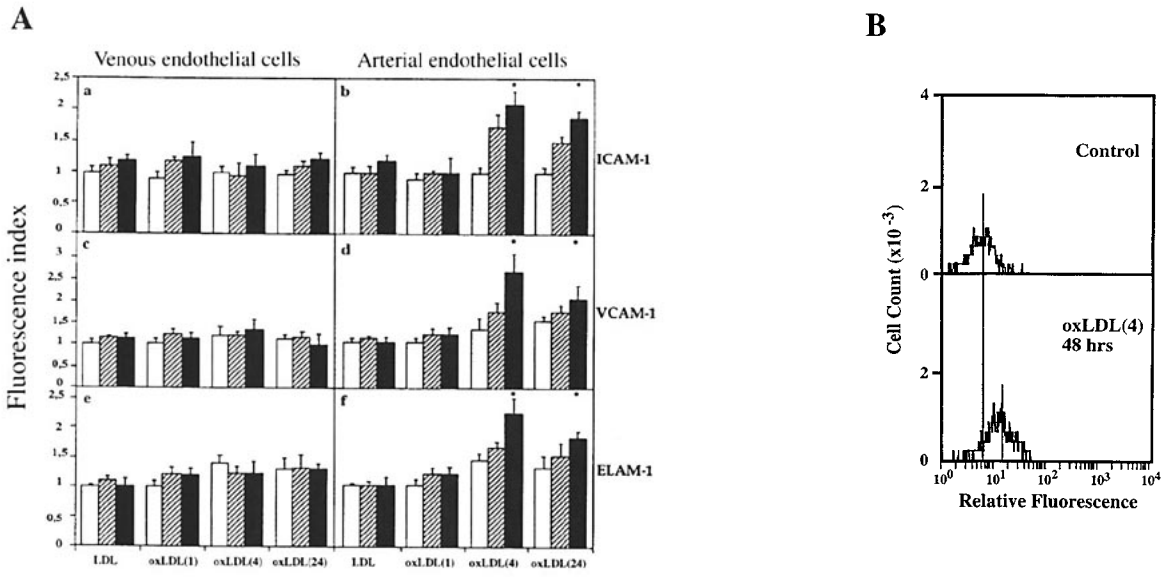

Fig. 3 FACS analysis of adhesion molecule expression on venous anf arterial EC after stress by LDL and oxLDL. Confluent arterial EC monolayers were treated with 80 μg/ml native, minimally (1), moderately (4) or highly (24) oxidized LDL from donors 1–3, respectively. Evaluation of fluorescence indices was performed as described in Fig. 2. Expression of ICAM-1, VCAM-1 and ELAM-1 in terms of mean values and standard deviations of 4 independent experiments are shown; (B) histogram for ELAM-1 expression after treatment with oxLDL(4) for 48 h (mean fluorescence intensity: control cells = 6.9; stressed cells = 17; fluorescence index = 2.45). □ 12 h; ▨ 24 h; ■ 48 h. *Significant difference from control cells, $P<0.05$.

not by LDL or oxLDL (not shown). Maximally induced expression could be detected at 4–8 h after stress and decreased to basal level after 24 h. Densitometric analysis revealed a 10-fold increase of Hsp60 expression after 8 h of heat or TNF-α treatment of EC (Fig. 4C). This time course of Hsp60 expression could be induced by all stressors and was similar in HAEC and HVEC. Hsp70 expression could only be detected after heat shock. Expression increased after 4 h and was still high at 12 and 24 h (not shown).

DISCUSSION

Adhesion molecule (ICAM-1, VCAM-1 ELAM-1) expression on EC is dramatically induced at sites of human atherosclerotic lesions (Springer 1994; Poston et al 1992; Printsevsa et al 1992; Van der Wal et al 1992). Overexpression of these molecules may play a role in adhesion and subsequent leucocyte transmigration into the subendothelial space, thus initiating a cascade of events leading to the formation of early atherosclerotic lesions. Earlier studies provided evidence that oxLDL exists in vivo in atherosclerotic lesions of humans and experimental animals (Ylä-Herttula et al 1989; Holvoet et al 1995), as well as in plasma of normal and atherosclerotic humans (Jürgens et al 1993; Holvoet et al 1995). Increased modified LDL concentration correlated with atherosclerosis and induced monocyte binding to the endothelium in vivo (Lehr et al 1993) and in vitro (Jeng et al 1993).

We found that oxLDL induced ICAM-1, VCAM-1 and ELAM-1 in arterial, but not venous, EC. The specific induction of VCAM-1 and ELAM-1 is dependent on pre-existing adhesion molecule transcripts, which are expressed after serum depletion in cell cultures. Addition of oxLDL to HAEC, cultivated in medium supplemented with 20% FCS showed no significant induction of adhesion molecule expression. Specific expression of VCAM-1 and ICAM-1 on HAEC after oxLDL exposure was recently described by Khan and co-workers (Khan et al 1995), who found that oxLDL did not induce adhesion molecule expression in HAEC when added to the cells alone, but enhanced TNF-α induced VCAM-1 and ICAM-1 expression. These results, and our own observations, suggest that the oxLDL-mediated induction of adhesion molecule expression depends on previously-expressed adhesion molecule mRNA in EC. This assumption is supported by recent studies by Ares and co-workers (Ares et al 1995) showing that oxLDL inhibited LPS-induced activation of NF-kB in human vascular smooth muscle cells. The transcription factor NF-kB binds to the 5′-regulatory sequences of a variety of genes, including those for ICAM-1, VCAM-1 and ELAM-1 and subsequently initates their transcription (Baeuerle, 1991). The mechanism of oxLDL action on EC is not yet known, and most studies have focussed on the role of lipid-hydroperoxide components of oxLDL, which affect ICAM-1 and VCAM-1, but not ELAM-1, expression in HAEC (Kume et al 1992;

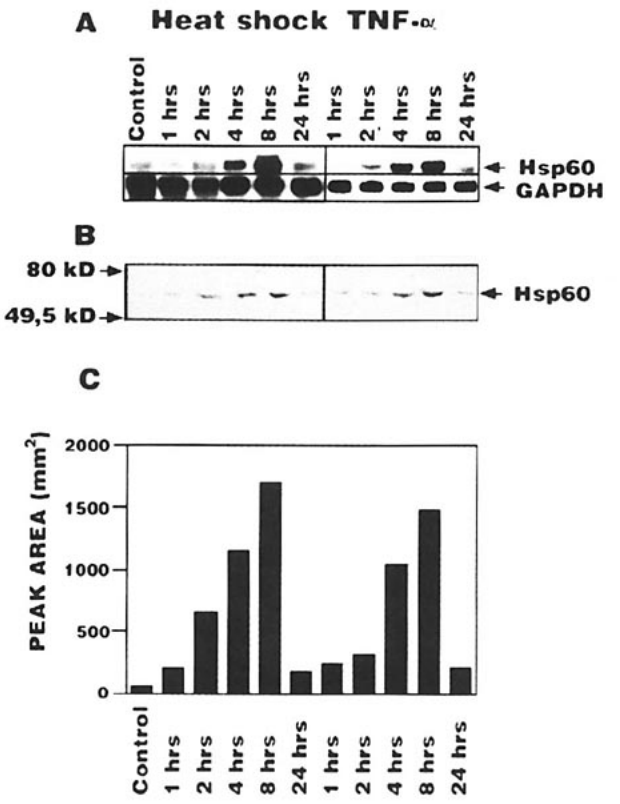

Fig. 4 Hsp60 expression in human EC. Expression of Hsp60 was analysed by Northern blot (A) and immunoblot (B). Proteins were separated by SDS-PAGE blotted on nitrocellulose filters and probed for Hsp60 expression. (C) The densitometric analysis of the blot shown in (B). This analysis revealed an at least 10-fold increase of Hsp60 production in HVEC 8 h after stress. This figure illustrates the Hsp60 expression of HVEC after stress by TNF-α and high temperature and is representative for IL-1, IFN-γ and for all venous and arterial EC-lines tested in this study.

Khan et al 1995). In contrast, we found that oxLDL in addition to VCAM-1 and ICAM-1 selectively induces ELAM-1 expression in HAEC. This contrary finding may result from the fact that the biochemical properties of oxLDL might vary between laboratories and preparations, and therefore must be defined very precisely as has been done in the present study. Our assumption is that modifications of oxLDL in both the lipid and protein portions are necessary for such a selective ELAM-1 induction.

Expression of adhesion molecules in EC is involved in the early steps of leucocyte emigration (Collins et al 1993; Springer 1994) and is chronically upregulated in EC overlaying atherosclerotic tissue (Van der Wal et al 1992). This in vivo observation, particulary for ELAM-1, contrasts with results in vitro, where ELAM-1 is rapidly downregulated after stimulation by cytokines and LPS (Bevilacqua 1993). We found that downregulation of ELAM-1 mRNA was much slower than downregulation of the ELAM-1 protein, and that induction of ELAM-1 by oxLDL depended on pre-existing ELAM-1 trancripts in HAEC. The ELAM-1 mRNA is apparently translated if oxLDL is applied to the cell culture, an oxLDL effect that could be responsible for the long lasting ELAM-1 protein expression observed in vivo. Studies by Witztum and coworkers indicate that LDL undergoes oxidative modification when incubated in vitro with EC (Witztum and Steinberg 1991). According to these studies it is tempting to suggest that HAEC exhibit a higher ability to modify LDL than HVEC. In our studies we could not detect an induction of adhesion molecule expression by LDL or minimally oxidized LDL in HAEC after 48 h so we assume that oxidation of LDL or oxLDL by HAEC is not the cause for the observed differences between HAEC and HVEC. However, the involvement of such processes in oxLDL mediated induction of adhesion molecules could not be excluded. In summary, our observations and the results of Khan et al. suggest that arterial EC are more sensitive to oxLDL treatment than HVEC, which could explain why leucocyte accumulation could only be observed in the intima of arteries.

In recent years, growing attention has been focussed on the functional differences between arterial and venous EC after cytokine treatment in vitro. As described previously, ICAM-1 and ELAM-1 expression shared a similar time course in HUVECs, HVECs and HAECs (Bevilacqua 1993; Klein et al 1994), but in contrast, VCAM-1 expression in HAECs is still controversial. Klein et al. demonstrated that HUVECs, HVECs and HAECs exhibited similar VCAM-1 expression patterns in response to IL-1β, TNF-α and LPS after 5 h. With respect to the time point, this is in agreement with our observations. In contrast, Hauser and collaborators (Hauser et al 1993) have shown that human arterial EC from iliac vessels did not express VCAM-1 after treatment with TNF-α, IL-4, IFN-γ or a combination of all three cytokines after 4 and 18 h. We found that VCAM-1 expression declined in venous EC after 12 h, whereas it is further induced in arterial EC at 24 h. Different responses of arterial EC to cytokines in vitro may be dependent on lifelong exposure to higher blood pressure in vivo, the number of passages that EC have undergone in vitro and the culture conditions used. Furthermore, it was demonstrated that even a weak increase of adhesion molecule expression in EC results in a 30–40% increase of monocyte binding to EC-monolayers (Jeng et al 1993). However, prolonged expression of VCAM-1 in arterial EC may be pivotal to leucocyte recruitment in regions of arterial vessels subjected to major hemodynamic stress (Li et al 1993). This is in agreement with in vivo studies demonstrating the lack of VCAM-1 expression in healthy arteries (Page et al 1992), but its chronic upregulation in EC overlaying atherosclerotic tissue (Li et al 1993).

In recent years, our laboratory suggested that one of the initiating events for atherosclerosis could be an immune reaction against Hsp60 (Wick et al 1992, 1995). Hsp exhibit considerable structural homology between bacterial and human proteins (Ferm et al 1992). Because of their phylogenetic conservation, it has been suggested that Hsp may provide a link between the immune response to infections and autoimmunity (Kume et al 1992). High local concentrations of Hsp60 could, therefore, trigger pre-existing Hsp60 reactive T-cells to initiate an inflammatory reaction (Lamb et al 1989). We found a constitutive expression of Hsp60 in HAEC and HVEC, that was further induced by cytokines, LPS or heat shock, but not by LDL or oxLDL. We further demonstrate that the regulation of expression of Hsp60 is similar in HAEC and HVEC in vitro. Expression of Hsp60 was observed in endothelium of fatty streaks and in macrophages and smooth muscle cells of thickened intimas, but not in the internal mammary or saphenous veins (Kleindienst et al 1993), that are notoriously used for coronary bypass operations. It was also shown that EC of large arterial vessels, which are subjected to stronger hemodynamic forces, pulsatile blood flow and pressure than venous EC, expressed Hsp60 (Kleindienst et al 1993), suggesting that in vivo combinations of various stress such as shear stress and elevated oxidized LDL levels alter the regulation of Hsp60 and adhesion molecule expression; or lower the threshold for induction of adhesion molecule synthesis in HAEC. The concomitant and long-term expression of Hsp60 and adhesion molecules in HAEC provide the prerequisite for the attachment and transmigration of Hsp65/60 reactive T-cells into the intima. Induction of atherosclerosis might be explained by cellular immunity to Hsp60 expressed by hemodynamically-stressed EC, leading to a chronic inflammatory process. Further studies on the activation of human EC by combinations of different stressors may provide new insights into the early steps of atherogenesis and could help us to understand the role of the immune system in this disease process.

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