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Role of Interleukin-4 in Atherosclerosis

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Vascular endothelial cell injury or dysfunction has been implicated in the onset and progression of cardiovascular diseases including atherosclerosis. A number of previous studies have demonstrated that the pro-oxidative and pro-inflammatory pathways within vascular endothelium play an important role in the initiation and progression of atherosclerosis. Recent evidence has provided compelling evidence to indicate that interleukin-4 (IL-4) can induce pro-inflammatory environment *via* oxidative stress-mediated up-regulation of inflammatory mediators such as cytokine, chemokine, and adhesion molecules in vascular endothelial cells. In addition, apoptotic cell death within vascular endothelium has been hypothesized to be involved in the development of atherosclerosis. Emerging evidence has demonstrated that IL-4 can induce apoptosis of human vascular endothelial cells through the caspase-3-dependent pathway, suggesting that IL-4 can increase endothelial cell turnover by accelerated apoptosis, the event which may cause the dysfunction of the vascular endothelium. These studies will have a high probability of revealing new directions that lead to the development of clinical strategies toward the prevention and/or treatment for individuals with inflammatory vascular diseases including atherosclerosis.

Key words: Interleukin-4, Atherosclerosis, Inflammation, Oxidative stress, Apoptotic cell death, Vascular endothelium, Molecular signaling pathways

INTRODUCTION

Activation or dysfunction of the vascular endothelium has been proposed to play a crucial role in the early events in the development of atherosclerosis (Gimbrone *et al.*, 2000; Toborek and Kaiser, 1999; Toborek *et al.*, 2002a). One of the most important functions of the vascular endothelium is to regulate inflammatory reactions. The development of inflammatory reactions is a normal defense mechanism in response to injury or activation of the vessel wall. The physiological significance of such reactions is to maintain and repair the normal structure and function of the vessel wall. Excessive inflammatory reactions, however, can lead to severe tissue damage and are associated with vascular pathophysiology, including the progression of atherosclerotic plaque formation (Toborek and Kaiser, 1999; Berliner *et al.*, 1995). It is now widely believed that atherosclerosis is an inflammatory disease of the vessel wall.

Inflammatory reactions in the vascular endothelium are primarily regulated through the production of inflammatory mediators (Lee *et al.*, 2004a; Ross, 1999). In fact, enhanced expression of pro-inflammatory cytokines (e.g., tumor necrosis factor-α; TNF-α), chemokines (e.g., monocyte chemoattractant protein-1; MCP-1), and adhesion molecules (e.g., intercellular cell adhesion molecule-1; ICAM-1 and vascular cell adhesion molecule-1; VCAM-1) in vascular endothelial cells and their close interactions facilitate recruiting and adhering inflammatory cells into the vessel wall, and thus stimulate transendothelial migration, which can be considered an early atherogenic process (Davies *et al.*, 1993; Reape and Groot, 1999). For example, ICAM-1 and VCAM-1 stimulate adhesion and transmigration of blood leukocytes onto and across the vascular endothelium. Both MCP-1 and, to a lesser extent, TNF-α are potent chemoattractive factors, which play a significant role in recruiting blood-born inflammatory cells into the vessel wall. In addition, TNF-α is a strong inducer of expression of a spectrum of pro-inflammatory mediators as well as of inflammatory reactions in vascular endothelium (Lukacs *et*

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al., 1995; Pober, 1998; Strieter *et al.*, 1989; Toborek *et al.*, 2002a).

It has been described that the expression of inflammatory mediators is regulated at the transcriptional level through activation of specific transcription factors (Lee *et al.*, 2001a, 2001b, 2003; Stanimirovic *et al.*, 2001; Toborek *et al.*, 2002b). The promoter region of inflammatory genes contains potential binding sites for a variety of transcription factors, including nuclear factor-κB (NF-κB), activator protein-1 (AP-1), cAMP responsive element-binding protein (CREB), SP-1, and signal transducers and activators of transcription (STAT-1 and STAT-6), etc. (Fig. 1). Compeling body of evidence has indicated the critical role of oxidative stress in molecular regulatory pathways leading to activation of these transcription factors and gene expression. It is now generally accepted that oxidative stress up-regulates the expression of pro-inflammatory mediator genes *via* activation of redox-responsive transcription factors. Indeed, activation of NF-κB and AP-1 are considered to be a part of a general regulation of a number of inflammatory gene expressions by cellular oxidative stress and/or intracellular glutathione levels (Arrigo, 1999; Bouloumie *et al.*, 1999; Lakshminarayanan *et al.*, 1996; Schreck *et al.*, 1992; Wung *et al.*, 1997). For example, expression of the inflammatory cytokine TNF-α gene is induced by increased oxidative stress through activation of AP-1 and NF-κB (Guha *et al.*, 2000; Lee *et al.*, 2001a, 2001b; Rahman and MacNee, 2000; Verhasselt *et al.*, 1998). In addition, recent evidences from our group and others have demonstrated that not only AP-1 and NF-κB but also other transcription factors, such as SP-1, CREB, STAT1, and STAT3, may belong to the family of transcription factors whose activity is regulated by oxidative stress and alterations in cellular redox status (Grosch and Kaina, 1999; Iwata *et al.*, 1997; Lee *et al.*, 2001c, 2001d, 2002, 2003; Madamanchi *et al.*, 2001; Simon *et al.*, 1998).

Interleukin-4 (IL-4) is a pleiotropic immunomodulatory cytokine secreted by T-helper 2 (TH2) lymphocytes, eosinophils, and mast cells (Paul, 1991; Rocken *et al.*, 1991). IL-4 is present at high levels in tissues of patients with chronic inflammatory diseases, where it may play a critical role in the disease progression. Indeed, elevated levels of IL-4 were detected in atherosclerotic lesions (Sasaguri *et al.*, 1998). Additionally, a growing body of evidence indicates that IL-4 may play a role in athero-

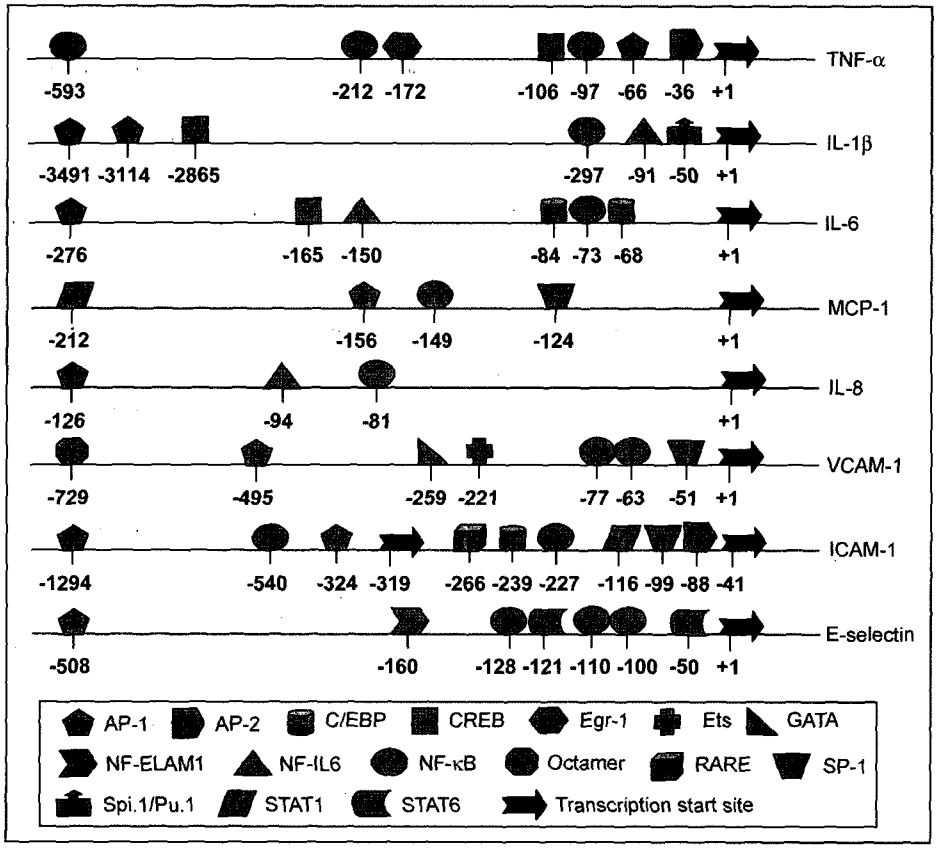


Fig. 1. Schematic representation of promoter regions of the human inflammatory genes showing the location of the transcription factor binding sites. Abbreviations: TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; IL-8, interleukin-8: VCAM-1. vascular cell adhesion molecule-1: ICAM-1. intercellular adhesion molecule.

genesis through induction of inflammatory responses, such as up-regulation of VCAM-1 (Galea *et al.*, 1991; Lee *et al.*, 2001d) and MCP-1 (Lee *et al.*, 2003; Rollins and Pober, 1991). IL-4 may also be considered as a pro-oxidative cytokine which can increase the oxidative potential of target cells (Brinckmann *et al.*, 1996; Lee *et al.*, 2001c, 2001d).

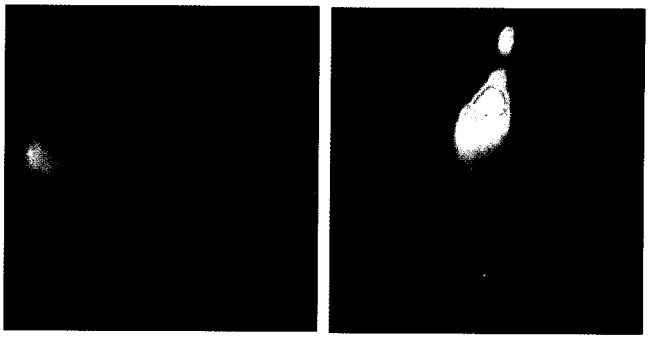
Although oxidative stress- and inflammation-induced endothelial cell dysfunction have been reported to play a critical role in the development of atherosclerosis, the detailed molecular signaling mechanisms underlying this process by pro-inflammatory cytokines and the potential involvement of antioxidant-sensitive mechanisms are not yet fully understood. This review will specifically focus on the pro-oxidative and pro-inflammatory mechanisms of vascular endothelial cell injury by IL-4.

THE PRO-OXIDATIVE AND PRO-INFLAMMATORY MECHANISMS OF IL-4-INDUCED VASCULAR ENDOTHELIAL CELL DYSFUNCTION

Induction of oxidative stress and pro-oxidative processes in IL-4-stimulated vascular endothelial cells

Oxidative stress has been implicated in atherogenesis (DiCorleto and Chisolm, 1986; Gimbrone *et al.*, 1990; Hennig *et al.*, 1996) and vascular endothelial cells are particularly sensitive to disturbances in the redox steady state (Hennig and Chow, 1988). It is also well known that oxidative stress may induce changes in cellular membrane structure, fluidity, transport, and antigenic characteristic as well as in disturbances in fibrinolytic pathway and prostacyclin synthesis (Hempel *et al.*, 1990; Holvoet and Collen, 1994). These abnormalities may ultimately contribute to endothelial cell injury, which is one of the earliest steps in the development of atherosclerotic lesions (Ross, 1993). In addition, it has been suggested that oxidative injury may regulate the expression of redox-sensitive genes, including those encoding for pro-inflammatory mediators (Ylä-Herttuala, 1992). Indeed, recent evidences from our group and others demonstrated that IL-4 can increase the oxidation potential of various cell types. For example, IL-4 treatment of human vascular endothelial cells (HUVEC) enhances the intracellular oxidizing potential, as indicated by an increase in 2',7'-dichlorofluorescein (DCF) fluorescence (Fig. 2) (Lee *et al.*, 2001d).

The reticulocyte-type 15-lipoxygenase (15-LO-I) is a lipid peroxidizing enzyme that converts free and/or esterified polyunsaturated fatty acids (e.g., linoleic acid or arachidonic acid) to hydroperoxy derivatives, such as 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13*S*-HPODE) and 15-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15-HPETE). In cellular systems 13-HPODE and 15-HPETE are rapidly reduced to 13-hydroxy-9*Z*,11*E*-octadecadienoic acid (13S-HODE) and 15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15-HETE), respectively. 15-LO-I has been implicated in atherogenesis (Feinmark and Cornicelli, 1997; Kühn and Chan, 1997) and this hypothesis is based on the observations that the purified enzyme is capable of oxidizing low density lipoproteins (LDL) to an atherogenic form (Belkner *et al.*, 1998; Sparrow *et al.*, 1998) and that 15-LO-I protein colocalizes in atherosclerotic lesions with epitopes of oxidized LDL (Ylä-Herttuala *et al.*, 1990). The recent finding that a specific 15-LO-I inhibitor attenuated the development of atherosclerosis in cholesterol-fed rabbits (Sendobry *et al.*, 1997) and that 12/15-LO-I knockout mice develop less pronounced atherosclerosis when crossed with apoE knockout mice (Cyrus *et al.*, 1999) appear to support this pro-atherogenic hypothesis. Because of the pathophysiological importance of 15-LO-I in atherosclerosis, the regulation of 15-LO-I expression has developed into a major field in lipoxygenase research. Several lines of experimental evidence indicate that IL-4 induces the expression of the 15-LO-I in human peripheral monocytes (Conrad *et al.*, 1992), alveolar macrophages (Levy *et al.*, 1993), colorectal carcinoma cells (Kamitani *et al.*, 1998), WI-26 pulmonary epithelial cells (Profita *et al.*, 1999), and the lung carcinoma cell line A549 (Brinckmann *et al.*, 1996). In addition, we have shown that IL-4 up-regulates the transcription of the 15-LO-I gene in human vascular endothelial cells and this process may involve the activation of a variety of transcription factors, such as STAT6, activator protein-2 (AP-2), GATA motif-binding transcription factor-1 (GATA-1), nuclear factor-1 (NF-1), and SP-1, for which putative binding sites exist in the 5'-



(A) (B)

Fig. 2. IL-4 treatment up-regulates the intracellular oxidizing reactions in human vascular endothelial cells as indicated by DCF fluorescence. HUVEC were either untreated (A) or treated with 10 ng/mL of IL-4 (B) for 3 h. Photomicrographs from confocal laser-scanning microscopy visualizing oxidative stress as DCF fluorescence emission. Blue color on the pseudocolor scale reflects low level of cellular oxidation, yellow intermediate, red high, and white the highest level of cellular oxidative stress (Lee *et al.*, 2001d).

flanking region of the human 15-LO-I gene (Lee *et al.*, 2001c).

Gene expression profile in IL-4-stimulated vascular endothelial cells

Microarray analysis is one of the most advanced and emerging molecular biological technologies, and it has been widely adopted for analyzing the global gene expression profiles *in vivo* and *in vitro* (Watson *et al.*, 1998; Schulze and Downward, 2001). Previous studies have demonstrated the potential of this technology for investigating molecular pathophysiological mechanisms involved in a variety of human diseases. In fact, microarray technology has been used as a novel experimental approach to analyze alterations in gene expression in cancer (Golub *et al.*, 1999), atherosclerosis (Hiltunen *et al.*, 2002), stroke (Bowler *et al.*, 2002), Alzheimer's disease (Ginsberg *et al.*, 2000), HIV infection (Geiss *et al.*, 2000), schizophrenia (Mirnics *et al.*, 2000), and muscular dystrophy (Chen *et al.*, 2000).

Recently, we performed microarray analysis using the Affymetrix GeneChip® Human Genome U133A Arrays and provided the first quantitative large-scale gene expression analysis of IL-4-stimulated human vascular endothelial cells (Lee *et al.*, 2004b). Our results identified 147 differentially regulated genes that are responsible for the regulation of inflammatory responses, apoptosis, signal transduction, transcription factors, metabolism, and several unknown function (Table I). Because IL-4 is involved in the early stages of atherogenesis, these results could contribute to a deeper understanding of fundamental insights of pathophysiological mechanisms involved in atherosclerosis at the level of gene expression and provide a foundation for development of therapeutic strategies for vascular diseases.

Table I. Summary of altered gene expression in human vascular endothelial cells treated with interleukin-4 (Lee *et al.*, 2004b)

Functional categories	Number of genes*	
	Up-regulation	Down-regulation
Adhesion molecules	8	1
Apoptosis	3	0
Cytokines, chemokines and receptors	2	3
Growth factors and receptors	0	4
Signal transduction	16	4
Transcription factors	16	4
Others (metabolism, etc.)	47	15
Unknown	14	10
Total	106	41

*Genes that are significantly up-regulated or down-regulated at least 2-fold changes compared to control cell cultures ($P < 0.05$).

Induction of adhesion molecule expression in IL-4-stimulated vascular endothelial cells

Vascular cell adhesion molecule-1 (VCAM-1) is a 110 kDa member of the immunoglobulin gene superfamily first described as a cytokine-inducible endothelial adhesion protein (Osborn *et al.*, 1989). It mediates cell-cell interactions *via* binding to its integrin counter receptor, i.e., very late antigen-4 (VLA-4), which may be involved in the recruitment of mononuclear leukocytes to the vascular lesions in early atherosclerosis (Elices *et al.*, 1990; Cybulsky and Gimbrone, 1991). Thus, VCAM-1 stimulates adhesion of lymphocyte and monocytes to the surface of the vascular endothelium (Libby and Galis, 1995). In addition, eosinophils and basophils, but not neutrophils, can bind to endothelial cells *via* VCAM-1-VLA-4 interaction (Schleimer *et al.*, 1992). This adhesion molecule is expressed primarily on endothelial cells; however, other cell types, both vascular and non-vascular cells, are also capable of expressing VCAM-1 (Barks *et al.*, 1997; Simmons *et al.*, 1992).

VCAM-1 expression in vascular endothelial cells has been widely known to be up-regulated by a variety of pro-inflammatory stimuli, such as interleukin-1β (IL-1β), TNF-α or lipopolysaccharide (LPS). Additionally, IL-4 was shown to increase the adhesiveness of leukocytes to human vascular endothelial cells *via* up-regulation of VCAM-1 (Galea *et al.*, 1991; Thornhill *et al.*, 1990). IL-4 can also synergize IL-1β-, TNF-α- or LPS-induced VCAM-1 gene expression in vascular endothelial cells (Barks *et al.*, 1997; Blease *et al.*, 1998; Masinovsky *et al.*, 1990). Functional analysis of the human VCAM-1 promoter demonstrated that several transcription factors including GATA, NF-κB, AP-1, interferon regulatory factor-1 (IRF-1), and SP-1 are associated with activation of VCAM-1 gene expression in response to IL-1β and TNF-α (Fig. 1) (Iademarco *et al.*, 1992; Iademarco *et al.*, 1993; Neish *et al.*, 1992; Neish *et al.*, 1995). Studies on the activity of the VCAM-1 gene promoter in endothelial cells have shown that up-regulation of VCAM-1 gene expression by IL-1β, TNF-α, and LPS depends on two adjacent κB sites located at positions -77 and -63 relative to the transcription initiation site (Iademarco *et al.*, 1992; Neish *et al.*, 1992). Moreover, AP-1 can mediate TNF-α-induced VCAM-1 expression interacting with NF-κB (Ahmad *et al.*, 1998). Despite our knowledge of molecular regulatory mechanisms of TNF-α or LPS-induced VCAM-1 overexpression in vascular endothelial cells, only limited information is currently available on possible mechanisms of IL-4-induced VCAM-1 gene expression. Surprisingly, several studies from our group and others have found that IL-4-mediated VCAM-1 expression is independent on both NF-κB and AP-1 activation (Lavie *et al.*, 1999; Lee *et al.*, 2001d; McCarty *et al.*, 1995; Wright *et al.*, 1999). It was reported that among known transcription factors which

have specific binding sites in the promoter region of the human VCAM-1 gene, only activation of SP-1 was observed when human vscular endothelial cells were treated with IL-4, suggesting that IL-4 up-regulates VCAM-1 gene expression in vascular endothelial cells at the transcriptional level *via* activation of SP-1 transcription factor. In contrast, NF-κB, AP-1, and IRF-1 do not appear to be involved in the signal transduction cascade. (Lee *et al.*, 2001d).

Another adhesion molecule regulated by IL-4 in vascular endothelial cells is E-selectin. E-selectin is selectively expressed on activated endothelial cells and plays a key role in mediating early leukocyte-endothelial interactions such as initial attachment and rolling during an inflammatory response. A compelling body of evidence indicates the crucial role of E-selectin in the pathogenesis of atherosclerosis. For example, the expression of E-selectin was detected in predominantly fibrous plaques and lipid-containing plaques of human coronary arteries (Davies *et al.*, 1993). It was also found that *in vivo* E-selectin up-regulation correlates the infiltration of polymorphonuclear leukocyte (PMN) and blood leukocyte (PBL) in a well-established inbred pig trafficking model (Binns *et al.*, 1996). It is well documented that E-selectin is a silent gene in vascular endothelium and rapidly up-regulated at the transcriptional level following exposure to a series of pro-inflammatory stimuli, such as TNF-α, IL-1β, and LPS (Luscinskas and Gimbrone, 1996; Tamaru and Narumi, 1999; Whelan, 1996). In contrast, treatment of endothelial cells with IL-4 suppresses IL-1β- or TNF-α-stimulated E-selectin gene transcription (Bennett *et al.*, 1997; Thornhill and Haskard, 1990; Whelan, 1996). Direct effects of IL-4 on E-selectin expression in human vascular endothelial cells, however, remain unclear. Recently, we have provided new evidence to strongly indicate that IL-4 could directly up-regulate mRNA and protein expression of E-selectin in human umbilical vein endothelial cells (HUVEC) (Lee *et al.*, 2004b). These results raise the possibility that E-selectin may play an important role in IL-4-mediated inflammatory pathways in vascular endothelium.

Induction of chemokine expression in IL-4-stimulated vascular endothelial cells

The recruitment of inflammatory cells such as monocytes and macrophages and their migration throughout the endothelium are thought to be critical early pathologic events in atherogenesis. These processes are directly promoted by chemokines, which are shown in recent studies to be closely related to the progression of atherosclerotic processes (Gu *et al.*, 1998, 1999; Rollins, 1997). Chemokines can be divided into two subfamilies, CXC and CC chemokines, based on structural and genetic considerations (Baggiolini *et al.*, 1997). Among a variety of chemokines, monocyte chemoattractant protein-1 (MCP-1) is of critical significance in the early stages of atherosclerosis. Human MCP-1, a 76-amino acid with an N-terminal pyroglutamic acid, is a member of the CC chemokine family and plays a crucial role in monocyte chemotaxis and transmigration. A compelling body of evidence indicates the potential role of MCP-1 in the pathogenesis of atherosclerosis. Both MCP-1 protein and mRNA expression have been detected in early atherosclerotic lesions by immunostaining, Northern blot analysis, and *in situ* hybridization (Nelken *et al.*, 1991; Seino *et al.*, 1995; Takeya *et al.*, 1993; Ylä-Herttuala *et al.*, 1991). Furthermore, MCP-1 deficiency significantly reduced atherosclerosis in low density lipoprotein (LDL) receptor-deficient mice fed a high cholesterol diet (Gu *et al.*, 1998). In a similar study, the selective absence of CCR2, the receptor for MCP-1, markedly decreased atherosclerotic lesion formation in apolipoprotein (apo) E-deficient mice (Boring *et al.*, 1998). On the other hand, Aiello *et al.* (1999) reported that overexpression of MCP-1 accelerated atherosclerosis in apoE-knockout mice. MCP-1 is expressed and released by a variety of cell types, including vascular endothelial cells, smooth muscle cells, monocytes/macrophages, and fibroblasts, in response to various stimuli such as inflammatory cytokines including IL-4, LPS, platelet-derived growth factor (PDGF), and interferon-γ (IFN-γ) (Bouloumie *et al.*, 1999; Rollins and Pober, 1991; Strieter *et al.*, 1989; Taubman *et al.*, 1992; Wung *et al.*, 1997; Zhou *et al.*, 1998). Although recent evidence indicates that IL-4 may stimulate the synthesis and secretion of MCP-1 in human vascular endothelial cells, the molecular regulatory mechanisms of MCP-1 expression by this cytokine is not yet fully understood.

The MCP-1 promoter has been shown to contain specific binding sequences for the redox-responsive transcription factors NF-κB and AP-1 (Shyy *et al.*, 1998). In fact, NF-κB and AP-1 have been known to be activated in response to alterations of cellular redox status in a wide range of cells, leading to the up-regulation of a number of pro-inflammatory genes including MCP-1 (Goebeler *et al.*, 2001; Shyy *et al.*, 1990; Ueda *et al.*, 1997; Wung *et al.*, 1997). However, our group has shown that treatment of HUVEC with IL-4 does not result in activation of NF-κB or AP-1 and induction of the inflammatory genes in response to IL-4 is independent of these transcription factors (Lee *et al.*, 2001c, 2001d). Therefore, the transcriptional regulation of MCP-1 expression by IL-4 in human vascular endothelial cells appears to be unique among a variety of biological systems.

Signal transducers and activators of transcription (STAT) transcription factors are latent cytoplasmic proteins that are activated by phosphorylation of a specific tyrosine residue and transduce a signal from a cytokine receptor. Phosphorylated STATs dimerize and rapidly translocate

into the nucleus, where they bind to specific DNA elements, activating transcription of target genes. To date, seven mammalian STAT family proteins have been identified as STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 and each protein has been shown to be activated by distinct cytokines (Ihle, 1996; Schindler and Dranell, 1995). Recently, Takeda and Akira (2000) have indicated the essential roles of each STAT family protein in cytokine-mediated biological responses through studies of gene targeted knockout mice, suggesting that STAT transcription factors act as critical intermediates in cytokine-dependent gene induction. Indeed, biological effects of IL-4 might be mediated by the activation of transcription factors of the STAT family. For example, it was demonstrated that IL-4 can specifically increase the STAT6-DNA binding activity, which appears to be a critical mechanism of IL-4-induced up-regulation of 15-lipoxygenase-I expression (Heydeck *et al.*, 1998; Lee *et al.*, 2001c; Schindler and Dranell, 1995). However, the possible relation between IL-4 and other STAT family proteins is not well defined. Specifically, the role of STAT1 activation in IL-4-induced alteration of endothelial cell metabolism remains unclear. Structural analysis of the 5'-flanking region of human MCP-1 gene reveals the existence of a potential binding site for STAT1 transcription factor (Fig. 1) (Shyy *et al.*, 1990; Zhou *et al.*, 1998). Recent studies from our group specifically focused on the role of STAT1 in IL-4-stimulated MCP-1 gene expression in human vascular endothelial cells (Lee *et al.*, 2003). It was found that dose-dependent increases in STAT1-DNA binding activity were detected in nuclear extracts prepared from HUVEC stimulated by IL-4 treatment. These results are in agreement with the report by Chang *et al.* (2000), who demonstrated that IL-4 can activate STAT1 in colon cancer cell lines, leading to growth inhibition. The role of STAT1 in MCP-1 gene expression was further confirmed by transient transfection experiments with the reporter plasmid constructs of the MCP-1 promoter (Lee *et al.*, 2003). Indeed, these results provide the first evidence that STAT1 signaling pathways may be critically involved in the transcriptional regulatory mechanisms of IL-4-induced MCP-1 expression.

Induction of cytokine expression in IL-4-stimulated vascular endothelial cells

Interleukin-6 (IL-6) is a multifunctional pro-inflammatory cytokine of 212 amino acid residues and produced by endothelial cells, mononuclear phagocytes, fibroblasts, activated T lymphocytes, and various neoplasms such as cardiac myxomas, bladder cancer, and cervical cancer. Previous studies have demonstrated that IL-6 plays a major role in the mediation of inflammatory and immune responses initiated by infection or injury. Indeed, elevated IL-6 levels have been reported in patients with a variety of diseases, including rheumatoid arthritis (Madhok *et al.*, 1993), inflammatory bowel disease (Hyams *et al.*, 1993), and malignancies such as myeloma, lymphomas, and ovarian cancer (Dunbar and Nienhuis, 1993; Kurzrock *et al.*, 1993; Watson *et al.*, 1993). Additionally, a compelling body of evidence indicates the crucial role of IL-6 in the pathogenesis of cardiovascular disease including atherosclerosis (Cesari *et al.*, 2003; Libby *et al.*, 2002; Ridker *et al.*, 2000a, 2000b; Ross, 1999). For example, the mRNA and protein expression of IL-6 have been detected in human atherosclerotic lesions (Kishikawa *et al.*, 1993; Seino *et al.*, 1994). IL-6 has also been found in the atherosclerotic plaques of apoE-knockout mice aorta and administration of exogenous IL-6 in apoE-knockout mice greatly exacerbated atherosclerotic lesion formation (Huber *et al.*, 1999; Sukovich *et al.*, 1998). Moreover, it has been shown that IL-6 stimulates the growth of vascular smooth muscle cells in a PDGF-dependent manner (Ikeda *et al.*, 1991).

IL-6 is rapidly up-regulated at the transcriptional level following exposure to a series of inflammatory stimuli, such as TNF-α, IL-1β, and LPS (Craig *et al.*, 2000; Kishimoto, 2005). Although recent evidence indicates that IL-4 synergistically amplifies the TNF-α-, IL-1β- or LPS-induced production of IL-6 protein in human vascular endothelial cells (Chen and Manning, 1996), the molecular basis for the induction of this cytokine by IL-4 has not been clearly elucidated. Our recent data showing that IL-4 significantly induced the expression of IL-6 mRNA and increased IL-6 production appear to be the first to document the stimulatory effect of IL-4 on IL-6 gene expression in human vascular endothelial cells (Lee *et al.*, 2004b). Functional analysis of the human IL-6 promoter demonstrated that several transcription factors including AP-1, NF-κB, cAMP responsive element-binding protein (CREB), and nuclear factor-IL6 (NF-IL6) are associated with induction of IL-6 gene expression (Fig. 1). However, detailed studies on the molecular signaling mechanisms of IL-6 expression in human vascular endothelial cells remain unclear and are to be further investigated.

Signaling mechanisms of IL-4-induced pro-inflammatory pathway in vascular endothelium

A number of previous studies have demonstrated that the binding of pro-inflammatory cytokines to their receptors triggers the mitogen-activated protein kinase (MAPK) signaling pathways that ultimately results in up-regulation of a wide variety of inflammatory genes, including cytokines, chemokines and adhesion molecules, *via* activation of inflammation-related transcription factors (Davis, 1993; Seger and Krebs, 1995). Three MAPK pathways with distinct regulation and functions have been described in mammalian cells; extracellular signal-regulated kinases

(ERK-1/2), c-Jun *N*-terminal kinases/stress-activated protein kinases (JNK/ SAPKs), and p38 MAPK (Pearson *et al.*, 2001). Among these, much attention has been focused on the activation and regulation of the p38 MAPK signaling pathway in inflammation research because p38 MAPK has been identified as key signaling molecules as therapeutic targets for a variety of inflammatory diseases (Badger *et al.*, 2000; Behr *et al.*, 2001; Collis *et al.*, 2001; Hull *et al.*, 2002; Ju *et al.*, 2002, 2003; Kawashima *et al.*, 2001; Kobayashi *et al.*, 2002; Kumar *et al.*, 2003; Ma *et al.*, 1999; Waetzig *et al.*, 2002). Compelling body of evidence from *in vivo* and *in vitro* studies has indicated that the p38 MAPK pathway is involved in the induction of pro-inflammatory mediators. For example, inhibitors of p38 MAPK have been shown to have anti-inflammatory effects through the inhibition of the expression of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6), cyclooxygenase 2 (COX2), and inducible nitric oxide synthase (iNOS) (Adams *et al.*, 2001; Badger *et al.*, 2000; Guan *et al.*, 1998; Han *et al.*, 1994; Lee *et al.*, 1994; Saccani *et al.*, 2002). Additionally, p38 MAPK has been implicated in the regulation of other pro-inflammatory mediators such as chemokines and adhesion molecules. Recent evidence shows that production of chemokines MCP-1 and interleukin-8 (IL-8) are mediated *via* p38 MAPK signaling pathway in response to inflammatory stimuli in monocytes/macrophages, vascular smooth muscle cells, and endothelial cells (Hall *et al.*, 2005; Suzuki *et al.*, 2004; Westra *et al.*, 2005). It was also demonstrated that pretreatment of activated endothelial cells with highly selective p38 MAPK inhibitors markedly reduced the mRNA and protein expression of adhesion molecules such as E-selectin, ICAM-1, and VCAM-1 (Ju *et al.*, 2003; Westra *et al.*, 2005), suggesting an important role for p38 MAPK in vascular endothelial inflammation and dysfunction. Furthermore, several compounds which specifically inhibit p38 MAPK, such as SB242235, RWJ-67657, VX-745, BIRB-976BS, and RO3201195, have been reported to advance to human clinical trials with promising pharmacokinetics and clinical activities (Fijen *et al.*, 2001; Kumar *et al.*, 2003). These previous preclinical and clinical studies strongly support the crucial role of p38 MAPK signaling pathway in inflammation and in the development of therapeutic strategies towards the treatment of inflammatory diseases including atherosclerosis.

It is well documented that two major signal transduction pathways, such as Janus kinase (JAK)-signal transducers and activators of transcription (STAT) and phosphoinositide-3 kinase (PI3K), are involved in the signaling cascade initiated by IL-4 (Pernis *et al.*, 1995). Emerging evidence has also established the p38 MAPK pathway as one element of the IL-4 signaling cascade. For example, Hunt *et al.* (2002) reported that IL-4 can activate p38 MAPK in the human primary monocytes as well as in the CT6 T-cell line and BA/F3 pro-B-cells. It was also shown that the p38 MAPK pathway is rapidly activated in splenic B lymphocytes by treatment with IL-4, and this activation is required for IL-4 induction of SOCS3 expression (Canfield *et al.*,

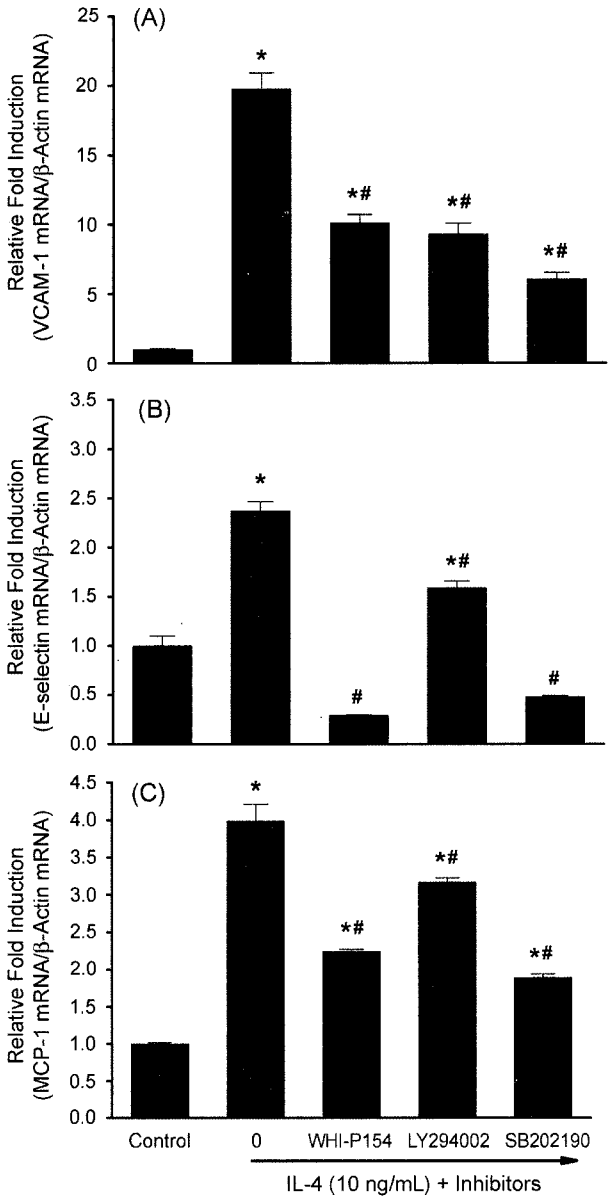


Fig. 3. Effects of selective inhibitors on IL-4-induced up-regulation of pro-inflammatory mediators in human vascular endothelial cells. HUVEC were pretreated with WHI-P154 (JAK inhibitor), LY294002 (PI3K inhibitor), or SB202190 (p38 MAPK inhibitor) for 1 h and treated with 10 ng/mL of IL-4 for 4 h. The mRNA levels of VCAM-1 (A), E-selectin (B), and MCP-1 (C) were determined by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Data shown are means ± SE of 4 determinations. *Statistically significant compared with the control group ($P<0.05$). #Values in the groups treated with IL-4 plus inhibitor are significantly different from the IL-4-treated group ($P<0.05$).

2005). Moreover, our most recent findings demonstrate that selective inhibitors for JAK/STAT, PI3K, and p38 MAPK signaling pathways significantly attenuate overexpression of pro-inflammatory mediators in IL-4-stimulated human vascular endothelial cells (Fig. 3; unpublished data). These data strongly support the crucial role of p38 MAPK in IL-4-mediated signaling cascade leading to up-regulation of pro-inflammatory mediators in vascular endothelium.

THE APOPTOTIC MECHANISMS OF IL-4-INDUCED VASCULAR ENDOTHELIAL CELL DYSFUNCTION

Endothelial cell apoptosis may be involved in the development and the progression of atherosclerosis (Bjorkerud and Bjorkerud, 1996; Bochaton-Piallat *et al.*, 1995; Han *et al.*, 1995; Isner *et al.*, 1995). For example, a high incidence of apoptotic cells is detected in atherosclerotic lesions, suggesting that apoptosis of endothelial cells might participate in remodeling of the vessel wall during atherogenesis (Han *et al.*, 1995). In addition, apoptotic smooth muscle cells are observed in balloon-injured arteries in rats (Bochaton-Piallat *et al.*, 1995; Han *et al.*, 1995) and in cholesterol-fed rabbits (Kochx *et al.*, 1996). However, the mechanisms that induce apoptosis in atherosclerotic lesions remain unclear.

Endothelial cells in atherosclerotic lesions may undergo apoptosis in response to the pro-inflammatory cytokines produced by activated macrophages and T lymphocytes as a consequence of the ongoing local immune and inflammatory response characteristic of atherogenesis (Geng and Libby, 1995). Previous studies have demonstrated that cytokines, such as IL-1β, TNF-α, and IFN-γ, can promote apoptosis of human vascular endothelial cells (Bennett *et al.*, 1994; Pohlman and Harlan, 1989; Polunovsky *et al.*, 1994). Additionally, our group has provided the first evidence that IL-4 can induce apoptosis of human vascular endothelial cells and the caspase-3-dependent pathway is critically involved in this process (Lee *et al.*, 2000), suggesting that IL-4 can increase endothelial cell turnover by accelerated apoptosis, the event which may alter the function of the vascular endothelium and thereby promote atherogenesis. Interestingly, it was shown that inhibition of protein synthesis may be required to promote complete DNA fragmentation of vascular endothelial cells. For example, a variety of pro-inflammatory stimuli including IL-1β, TNF-α, IL-4, and LPS have been reported to induce endothelial cell apoptosis in the presence of a protein synthesis inhibitor (Gottlieb *et al.*, 1994; Lee *et al.*, 2000; Pohlman and Harlan, 1989; Polunovsky *et al.*, 1994). Results of these studies suggest that inducible or constitutive cytoprotective proteins control endothelial cell survival. However, such proteins which may control IL-4-induced apoptosis of vascular endothelial cells remain yet to be characterized.

CONCLUSION

Oxidative stress-mediated pro-inflammatory environment and apoptotic cell death within vascular endothelium in

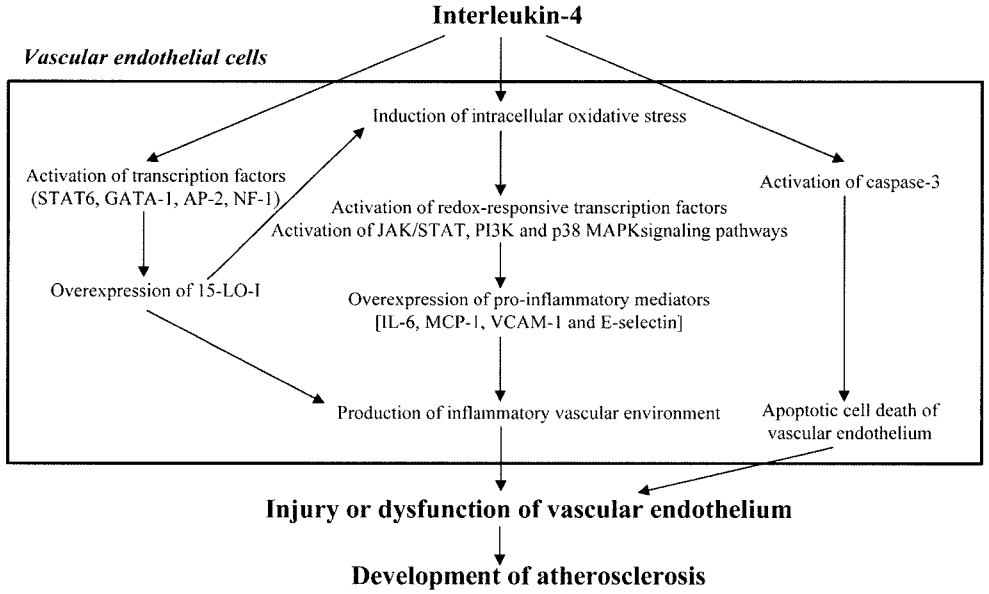


Fig. 4. Schematic diagram of the molecular signaling mechanisms of IL-4-induced vascular endothelial cell dysfunction and development of atherosclerosis. Abbreviations: JAK/STAT, Janus kinase-signal transducers and activators of transcription; PI3K, phosphoinositide-3 kinase; p38 MAPK, p38 mitogen-activated protein kinase; 15-LO-I, 15-lipoxygenase-I; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cell adhesion molecule-1.

response to a variety of extracellular stimuli such as TNF-α, IL-1β, and LPS have been known as major mechanisms of vascular injury leading to the development of atherosclerosis. It appears that IL-4 can be in part responsible for these effects (Fig. 4). Indeed, evidence indicates that IL-4 can induce intracellular oxidative stress, overexpression of pro-inflammatory mediators including cytokine, chemokine and adhesion molecules, and inflammatory reactions in vascular endothelial cells. In addition, IL-4 can increase apoptotic cell death of vascular endothelium. More importantly, recent results showing potential involvement of antioxidant-sensitive mechanisms and p38 MAPK-mediated signaling pathway in this process will have significant clinical implications for the development of therapeutic drugs for atherosclerosis specifically targeted against pro-oxidative and pro-inflammatory pathways.

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