Genes affecting triglyceride metabolism : from steatosis to lipodystrophy
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Chapter 2

Gene Expression in Atherogenesis

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Summary

It is conceivable that the extent and spatio-temporal expression of dozens or even a few hundred genes are significantly altered during the development and progression of atherosclerosis as compared to normal circumstances. Differential gene expression in vascular cells and in blood cells, due to gene-gene and gene-environment interactions can be considered the molecular basis for this disease. To comprehend the coherence of the complex genetic response to systemic and local atherosclerotic challenges, one needs accessible high through-put technologies to analyze a panel of differentially expressed genes and to describe the interactions between and among their gene products. Fortunately, new technologies have been developed which allow a complete inventory of differential gene expression, i.e. DD/RT-PCR, SAGE and DNA micro-array. The initial data on the application of these technologies in cardiovascular research are now being reported. This review summarizes a number of key observations. Special attention is paid to a few central transcription factors which are differentially expressed in endothelial cells, smooth muscle cells or monocytes/macrophages. Recent data on the role of nuclear factor-κB (NF-κB) and peroxisome proliferation-activating receptors (PPARs) are discussed. Like the PPARs, the NGFI-B subfamily of orphan receptors (TR3, MINOR and NOT) also belongs to the steroid/thyroid hormone receptor superfamily of transcription factors. We report that this subfamily is specifically induced in a sub-population of neointimal smooth muscle cells. Furthermore, intriguing new data implicating the Sp/XKLF family of transcription factors in cell-cell communication and maintenance of the atherogenic phenotype are mentioned. A member of the Sp/XKLF family, the shear stress-regulated lung Krüppel-like factor (LKLF) is speculated to be instrumental for the communication between endothelial cells and smooth muscle cells. Taken together, the expectation is that the fundamental knowledge obtained on atherogenesis and the data that will be acquired during the coming decade with the new, powerful high through-put methodologies will lead to novel modalities to treat patients suffering from cardiovascular disease. In view of the phenotypic changes of vascular and blood-borne cells during atherogenesis, therapeutic interventions likely will focus on reversal of an acquired phenotype by gene therapy approach or by using specific drugs which interfere with aberrant gene expression.
Introduction

Atherosclerosis is a complex, chronic inflammatory disease of the arterial vessel wall which is manifested at predilected locations of the vasculature [reviewed in (1-3)]. Accordingly, the current opinion is that this disease is caused by both local and systemic risk factors. Local risk factors are determined by the geometry of the vessels and the view has emerged that bifurcations, branch points and curvatures intrinsically cause irregularities of the otherwise uniform laminar shear-stress (4-6). Areas of turbulent flow, resulting in abrupt transitions of low and high shear stress, are particularly prone to develop atherosclerotic lesions. The formation of atherosclerotic lesions is considered as the basis for the etiology of most pathological, cardiovascular events such as coronary heart disease (CHD) (7, 8). The systemic risk factors which cause these diseases, have been thoroughly defined by epidemiological studies and traditionally include high plasma cholesterol, smoking, male sex, advanced age, diabetes mellitus, overweight and high blood pressure (9). Novel risk factors have now been introduced and are under investigation such as hyperhomocysteinemia, impaired fibrinolysis, lipoprotein (a), small dense low-density lipoprotein (LDL) and inflammatory markers (10). Ever since the epidemiological studies identified the risk factors for CHD, scientists have been challenged to translate these risk factors into patho-biological pathways and define a molecular basis for atherosclerosis.

Among the patho-biological processes involved in atherosclerosis, the importance of lipoprotein metabolism in initiation and progression of atherosclerosis has been established by human as well as animal studies. The earliest form of atherosclerosis, called “fatty streaks”, could start in infancy and these lesions contain inflammatory cells, such as macrophages and lymphocytes, in addition to accumulated lipids. This has led to the generally accepted hypothesis that atherosclerosis is a chronic inflammatory disease of the vessel wall [reviewed in (1-3)]. According to this hypothesis, endothelial cell dysfunction upon different “injuries”, such as oxidized low-density lipoprotein (ox-LDL), is the first step in atherogenesis. The dysfunction leads to increased permeability and to an increased adhesion of leukocytes and platelets to the endothelium. The interaction between endothelial cells and leukocytes or platelets is mediated by selectins, surface-exposed receptors, that bind to defined ligands on the indicated blood cells (11). Subsequently, leukocytes (e.g. monocytes and T cells) transmigrate through the endothelium by transiently disrupting the gap junctions and the tight junctions between adjacent endothelial cells [reviewed in (12)]. In the sub-endothelial space, monocytes differentiate into macrophages and get engaged in digesting large amounts of (modified) lipoprotein particles. Consequently, these cells turn into lipid-laden macrophages (“foam-cells”). The monocytes/macrophages release cytokines and chemokines which will reach the medial smooth muscle cells and strongly affect their fate. Under normal conditions, smooth muscle cells are specialized to provide elasticity and contractability to the arterial vessel wall. However, due to the monocyte/macrophage-derived factors, smooth muscle cells leave their medial location, traverse the internal elastic lamina and migrate into the intima. Subsequently or simultaneously, smooth muscle cells change from a contractile...
(differentiated) phenotype into a non-contractile, proliferative (partially dedifferentiated) phenotype, reminiscent of transitions observed during early fetal development (13-15). In addition, dedifferentiated smooth muscle cells synthesize large amounts of extracellular matrix proteins, ultimately resulting in a fibrous lesion that may partially obstruct the circulation. The susceptibility of the lesion with respect to rupture is dependent on the lipid content of the plaque or, more precisely, on the relative amount of fibrous material versus atheromatous core.

Each of the distinct steps in atherogenesis is driven by a set of genes which is expressed by a variety of cells as outlined above. Although it has been announced that the nucleotide sequence of the entire human genome has now been resolved, it is obvious that only a fraction of the genes and gene products has been characterized. It is conceivable that, among the tremendous number of unidentified genes, a subset will be responsible for atherogenesis. Hence, we are faced with the challenge of identifying these genes and elucidating their function, in order to get a firm understanding of the molecular basis of this multifactorial disease. Fortunately, during the past decade, novel high through-put technologies have been developed which provide unbiased approaches to make an inventory of differentially expressed genes. These technologies have recently been applied in cardiovascular research by our laboratory and by others and will be briefly outlined in the next section.

There is no doubt that genes expressed in the liver and the intestine have an important role in lipid metabolism and in atherogenesis. However, this review focuses on genes expressed by the vascular cells and, consequently, we will refrain from discussing genes expressed in the aforementioned organs. We have chosen to discuss individually the contribution of endothelial cells, smooth muscle cells and monocytes/macrophages to atherogenesis. It is expected that, in the near future, integration of the results obtained for separate cell types will be done e.g. by employing co-culture systems of vascular cells as has been described previously (16, 17). Furthermore, in view of the molecular complexity of the patho-physiology of atherogenesis, we have restricted this review to a discussion of recent data on the contribution of specific (families of) transcription factors which regulate genes which are possibly causative determinants for initiation and progression of atherosclerosis. In particular, recent data on the role of nuclear factor-κB (NF-κB), peroxisome proliferation-activating receptors (PPARs) and the Sp/XKLF family of zinc finger proteins will be discussed. It may be hypothesized that interfering with the action of these transcription factors, or with up-stream or down-stream events related to these factors, may provide opportunities to develop new diagnostic tools and therapeutic agents and modalities either to prevent the formation of lesions or to induce their regression.
High Through-put Techniques to Monitor Differential Gene Expression

Three major techniques have been developed during the last decade which allow high through-put analysis of differential gene expression in complex organisms. These techniques are denoted differential display of randomly-primed mRNA by RT-PCR (DD/RT-PCR) (18, 19), serial analysis of gene expression (SAGE) (20) and DNA micro-array (21). Our laboratory has implemented and validated each of these techniques and we believe that each of them has its specific merits and drawbacks. For example, in principle, DD/RT-PCR reveals differential expression of both high and low expressing genes, but does not provide quantitative insight into the steady-state concentration of mRNAs. Furthermore, DD/RT-PCR is based on the use of anchored- and arbitrary primers and the number of primer combinations determines the actual “coverage” of the collection of different mRNAs (denoted “transcriptome”). For example, usually 144 DD/RT-PCR reactions are performed with 12 anchored- and 12 arbitrary primers, theoretically comprising about 80% of the mRNA profile, meaning that the remaining part of the transcriptome is lacking (19). SAGE does provide quantitative data on the level of (differential) mRNA synthesis, but obtaining a collection of statistically relevant data depends on the availability of large-scale DNA sequencing facilities. Since both techniques rely on amplification of mRNA by RT-PCR, they are both very sensitive. DNA micro-array is based on hybridization of radiolabeled or fluorescently labeled cDNA preparations to either filter- or glass-immobilized single-stranded cDNA or a series of gene-specific and control oligonucleotides. The amplified, labeled cDNA preparation should be a qualitative and quantitative representation of the mRNA profile of a given tissue, cell type or mixture of different cells and, hence, is highly heterogeneous in terms of amount of distinct mRNAs. As a consequence, the sensitivity of hybridization, which is determined among other things by the concentration of the hybridizing counterpart (probe), might be modest for genes that are expressed at a very low mRNA level. On the other hand, DNA micro-array is a genuine high through-put technique which in principle allows a “genome-wide” comparison of, for example, gene expression of a chosen cell type/tissue under different conditions or of two different cell types under the same condition. As an illustration of large-scale gene expression profiling, tumor endothelium has been compared with normal endothelium, using SAGE (22). Undoubtedly, similar studies will be undertaken to compare the transcriptome of vascular cells under atherosclerotic circumstances and that of their healthy counterparts.

We have recently designed and applied a combined protocol of DD/RT-PCR and DNA micro-array. Initially, DD/RT-PCR had been employed to isolate and identify genes differentially expressed in cultured human vascular endothelial cells and smooth muscle cells that were subjected to a strong atherosclerotic stimulus (23,24). In addition, we also performed an extensive SAGE to delineate genes that are specifically induced or repressed upon activation of cultured human smooth muscle cells (25). Together with cDNAs, corresponding to genes differentially expressed upon the transition of cultured human monocytes into macrophages (V. Sier-Ferreira, B.M.M, van den Berg, H. Pannekoek, unpublished data), we composed a
limited, “custom” human vascular DNA micro-array which contains about 350 cDNAs (26). These arrays are currently used to analyze expression of this pre-selected set of genes in specimens collected during major vascular surgery or in vascular material that has been obtained from organ donors (27). Obviously, the bioinformatics required to “read” these custom arrays is relatively straightforward as compared to genome-wide screens which will be available in due time.

Vascular Cells

The non-atherosclerotic (“healthy”) vessel wall contains only two different cell types, endothelial cells and smooth muscle cells, each of which has a well-defined role. Under these conditions, the monocellular endothelial layer forms a border that permits transport of nutrients to the sub-endothelial space. The endothelial cells synthesize products that are important to maintain the contractile function of the underlying smooth muscle cells. This communicative function is attributed in particular to small molecules like prostanoids, endothelins and nitric oxide (NO). Indications are at hand, however, suggesting that additional pathways are also involved in the communication between endothelial cells and smooth muscle cells. Hence, it is foreseen that future studies will focus on the identification of these molecules and the elucidation of their function. In this respect, the seminal studies on the interplay between cardiac microvascular endothelial cells and cardiomyocytes may serve as an excellent example (28-31). Here, it was demonstrated that differential (regional) vascular-bed specific expression of von Willebrand was mediated by signal transduction initiated by way of hetero-dimeric platelet-derived growth factor AB (PDGF AB). Remarkably, endothelial cells and cardiomyocytes both contribute to the generation of PDGF AB. In the absence of cardiomyocytes, cardiac microvascular endothelial cells exclusively synthesize PDGF A. Co-culturing of these two different cell types results in the induction by cardiomyocytes of endothelial PDGF B synthesis, allowing the subsequent generation of heterodimeric PDGF AB. PDGF AB is a ligand for the so-called α-receptor and the interaction between ligand and receptor ultimately leads to von Willebrand factor synthesis. This example elegantly illustrates the importance of cell-cell communication and provides a mechanistic explanation for regional-specific gene expression. In the following paragraph, we will present a similar, though less well understood, example of communication between the endothelial cells and smooth muscle cells.

The atherosclerotic vessel wall harbors inflammatory cells, notably monocytes and T cells, in addition to endothelial cells and smooth muscle cells. As indicated before, these cells transmigrate through the endothelial cell layer by interaction with the proteins that constitute the gap junctions and the tight junctions between adjacent endothelial cells (reviewed in [12]). Communication between monocytes, which differentiate in the vessel wall into macrophages, and smooth muscle cells, is mediated by cytokines and chemokines which are not synthesized at these locations under non-atherosclerotic circumstances (reviewed in [1-3]). Hence, the accumulation of lipid-laden macrophages in the vessel wall constitutes a hallmark
of this disease and conceivably forms a decisive factor in the development of a rupture-prone plaque. Taken together, the picture is emerging that dysregulation of gene expression of different cell types and, consequently, altered communication between these cells is the molecular basis for this disease. However, since alterations of cell-cell communication in atherogenesis are still poorly understood, we will discuss a number of aspects of differential gene expression of “isolated” vascular cells.

Endothelial Cells

The endothelium covers the luminal side of the entire cardiovascular system and has been considered as a distinct organ. It covers approximately 700 m², weighs 1.5 kg and regulates many processes (32, 33). It forms a non-thrombogenic, non-adhesive layer and regulates vascular tone. It is now generally accepted that endothelial dysfunction is the first step in atherosclerosis [reviewed in (1-3)]. Endothelial dysfunction refers to impaired biological processes in endothelial cells, leading to increased adhesiveness to monocytes, increased permeability, procoagulant properties and changes in vascular tone. However, in clinical settings endothelial dysfunction has been used to describe the impaired nitric oxide (NO) derived vasodilatation. Endothelial cells are exposed to a variety of biochemical and biomechanical stimuli, such as circulating LDL, inflammatory cytokines, fluid shear stress and cyclic stretch (4-6,34). Since it is generally assumed that atherosclerosis is due to an interplay between genes and environmental factors, individuals should react differently to these stimuli. Accordingly, experiments with mice of different genetic backgrounds illustrate a different sensitivity towards atherogenic stimuli and differences in the gene expression pattern of their endothelial cells (35). By using two strains of mice, one resistant (C3H/HeJ) and the other susceptible (C57BL/6J) to diet-induced atherosclerosis, it was shown that cultured endothelial cells isolated from these strains of mice reacted differently to stimuli. Minimally-modified LDL induced higher levels of inflammatory genes in cultured endothelial cells derived from the C57BL/6J mice than in those derived from C3H/HeJ mice. This study not only explains, at least partly, the differences in susceptibility of these mice, but also illustrates that factors acting in the vessel wall could protect against diet-induced atherosclerosis. Finally, these observations imply that altering the expression profile of vascular cells by, for instance, gene therapy or local drug delivery, might be a useful way to treat atherosclerosis.

Since atherosclerosis is considered a chronic inflammatory disease of the vessel wall, it is obvious that special attention should be given to the transcription factor, nuclear factor κB (NF-κB). NF-κB is a heterodimeric, DNA-binding protein, being one of the key regulators of gene expression in the vessel wall and plays a coordinating role in inflammation. Its role in atherosclerosis is emphasized by the notion that the activated form is exclusively encountered in vascular lesions and not in healthy tissue (36). The mode of activation of NF-κB has been elucidated in detail. It involves the activation of a dimeric IκB kinase complex which phosphorylates the NF-κB inhibitor IκB, leading to its subsequent ubiquitination and degradation and, ultimately, release of NF-κB (37). Free NF-κB dimers then translocate to the
nucleus where the assembled transcription factor regulates the mRNA synthesis of target genes. Recently, it was shown in a pig model that a high cholesterol diet resulted in higher expression of the activated NF-κB in the coronary vasculature (38). In view of the mediating role of activated NF-κB in inflammation, it is conceivable that high cholesterol levels cause inflammation of the vessel wall, as evidenced by the presence of activated NF-κB. Similarly, tumor necrosis factor alpha (TNF-α) is a potent inflammatory cytokine which also activates the NF-κB signaling pathway.

Although the role of the NF-κB pathway in atherogenesis is undisputed, it is obvious that many other known and unknown genes play a crucial role in initiation and progression of this disease. To identify novel and known genes expressed by endothelial cells and involved in atherosclerosis, differential display of gene expression by randomly primed mRNA, using RT-PCR was employed (DD/RT-PCR; 18, 19). The mRNA profile of quiescent human umbilical vein endothelial cells (HUVEC) was compared with that of TNF-α-stimulated HUVEC, resembling the pro-atherogenic, inflammatory phenotype (23). A total of 106 differentially expressed gene fragments were identified and these included 22 known genes (Table 1). Many of these known genes have previously been implicated in atherosclerosis, such as monocyte chemoattractant protein-1 (MCP-1) and interleukin 8 (IL-8), showing the validity of this approach. On the other hand, as noted before, DD/RT-PCR analysis does not cover the entire transcriptome since a number of anticipated, differentially expressed mRNAs were not encountered. Notably, we did not detect a differential display (DD) fragment corresponding to transcripts of e.g. NF-κB nor to plasminogen activator inhibitor 1 (PAI-1). The latter transcript was, however, encountered in a rather limited SAGE of resting HUVEC versus cells activated with TNF-α-containing conditioned medium of ox-LDL-activated cultured monocytes (39). This observation demonstrates that it is worthwhile to simultaneously explore different (complementary) high through-put technologies to make an inventory of genes which are differentially expressed. Some of the remaining unknown endothelial cell DD gene fragments (37 out of 84) are present in public databases (dbEST), indicating that these genes are indeed expressed and represent genuine transcripts. A subset of these ESTs is currently under investigation to explore their role in vascular disease. Finally, it should be noted from the catalogue of differentially expressed genes (Table 1) that activation of endothelial cells with a “strong” atherosclerotic stimulus, i.e. TNF-α or TNF-α-containing conditioned medium of ox-LDL-treated monocytes, results in alterations of an impressive number of diverse processes. These include haemostasis, leukocyte trafficking, regulation of transcription, protection against oxidation, cell shape and cell cycle, signal transduction and apoptosis.

Despite the importance of systemic factors for its initiation and progression, atherosclerosis develops at preselected sites in the arterial tree, such as the outer edges of vessel bifurcations, branch points and curvatures (4-6, 34). In these susceptible areas, laminar blood flow is disturbed resulting in an altered shear stress. It has been postulated that the gene expression pattern of the endothelial cells is modulated at the transition of high to low shear stress, leading to a focal atherogenic phenotype of these cells (40-42). Indeed, in vitro studies have shown that the mRNA profile of cultured endothelial cells changes upon applying
shear stress. For instance, endothelial NO-synthase (eNOS), an enzyme that catalyses the production of the vasodilator NO, is increased in vitro by fluid shear stress (43). En-face examination of endothelial-cell surfaces in human thoracic aortas revealed a pro-atherogenic phenotype in areas with low shear stress (accumulation of sub-endothelial macrophages and lymphocytes, irregular endothelial morphology with denuded regions) (44). Recently, evidence has been acquired that the effects of low shear stress are also transmitted through the NF-κB pathway (45). These investigators showed that areas with disturbed flow (high probability) in mice aorta display a higher expression level of NF-κB as compared to areas with high shear stress (low probability region). Upon feeding an atherogenic diet to these mice,

Table I Cumulative data on affected genes, the corresponding Genbank (GB) accession number and affected processes of cytokine-stimulated endothelial cells

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<tr>
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<td>GM-CSF</td>
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</tr>
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<td>cellular interactions</td>
</tr>
<tr>
<td>RANTES</td>
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<td>ribos. proteinSII</td>
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<td>translation</td>
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<td>apoL3 = CG12-1*</td>
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NF-κB was predominantly activated in those high probability areas, leading to the conclusion that these areas are primed for activation by various stimuli, whereas the low probability regions with high shear stress are apparently resistant to diet-induced atherosclerosis. These studies show that high shear stress indeed induces a quiescent, anti-atherogenic phenotype of the endothelial cells and it is speculated that this is accomplished by interfering with inflammatory pathways.

How do the endothelial cells “sense” these biomechanical forces and how do they change their expression profile? Unfortunately, neither the receptor(s) for shear stress nor the downstream signaling pathway(s) have yet been clarified. Recently, however, we found by using commercial DNA macro-arrays that, among other genes, lung Krüppel-like factor (LKLF) was up-regulated by shear stress (R. J. Dekker, H. Pannekoek and A. J. G. Horrevoets, unpublished data). LKLF is a zinc finger transcription factor that belongs to the Sp/XKLF family (reviewed in ref 46). Most of these proteins have been reported to promote transcription, but some members of this family exert an inhibitory effect. LKLF was previously shown to be expressed in naïve T cells and was rapidly repressed after T cell activation (47). In that study, it was concluded that LKLF is required to program the quiescent state of T cells and to maintain their viability in the peripheral lymphoid organs. LKLF is also highly expressed in vascular endothelial cells between E9.5 and E12.5 of mouse embryogenesis, a critical time for vessel wall stabilization (48). It turned out that LKLF-deficient mice die in utero due to haemorrhages that are caused by severe defects in vessel wall integrity. Apparently, LKLF−/− mice are unable to form an organized tunica media. However, we found by in situ hybridization that LKLF is exclusively expressed in endothelial cells of the human aorta and it seems to be confined to areas with high shear stress. No expression was observed in the media, presumably since smooth muscle cells do not synthesize LKLF (R. J. Dekker, H. Pannekoek and A. J. G. Horrevoets, unpublished data). Consequently, we propose that these observations can be reconciled by assuming that secreted endothelial factors synthesized downstream of LKLF may interact with the smooth muscle cells and are involved in the organization of the media. Interestingly, LKLF was down-regulated by cultured HUVECs which were treated with TNF-α, suggesting that the NF-κB signaling pathway counteracts gene regulation by LKLF.

Smooth Muscle Cells

Smooth muscle cells do not terminally differentiate and do exhibit different phenotypes, dependent on the presence of specific growth factors and cytokines, secreted by endothelial cells and surrounding macrophages. Contractile (differentiated) smooth muscle cells are quiescent and regulate the vascular tone (13). In contrast, under atherosclerotic conditions, smooth muscle cells respond to growth factors and cytokines and dedifferentiate into a “synthetic”, proliferative phenotype. These cells are able to migrate from the media into the intima and to produce large amounts of extracellular matrix constituents (13, 14). Consequently, a substantial part of the atherosclerotic, neointimal, lesion is formed by smooth
muscle cells and their matrix proteins. Conversely, apoptosis of smooth muscle cells would decrease the extent of lesion formation and promotion of this process may thus constitute a promising strategy to relieve obstruction of the circulation.

The alterations in gene expression, resulting from the transition of quiescent to activated smooth muscle cells, are largely unknown. At present, only a few genes have been identified that are specifically induced in cultured smooth muscle cells upon applying an atherosclerotic stimulus (indicated below). To create an unbiased inventory of differential gene expression of resting versus activated cultured, human smooth muscle cells, we performed both DD/RT-PCR (24) and SAGE (25). Here, the results obtained with DD/RT-PCR will be briefly summarized (Table 2). By applying DD/RT-PCR, using 12 different anchored primers and 12 different arbitrary primers, we identified 40 genes that are induced in smooth muscle cells upon activation with the conditioned medium of ox-LDL-treated cultured human monocytes. Ten differentially expressed genes have a known function, whereas 30 are novel genes of unknown function of which only a few are encountered in dbEST. Among the 10 known genes, some have been reported to be involved in atherogenesis, notably IL-8, intercellular adhesion molecule-1 (ICAM-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Interestingly, a comparison of the differential gene expression profile of cultured endothelial cells and smooth muscle cells, exposed to the same atherogenic stimulus, reveals that these cell types respond via a virtually unique inRNA repertoire. Except for a few genes [IL-8, GM-CSF and the human inhibitor of apoptosis protein-1 (hIAP-1)] both the inRNAs of differentially expressed known genes and the ESTs are apparently specific for each of these cell types.

As in previous sections, we will focus here on differential expression of transcription factors in smooth muscle cells. Interestingly, we found that the central transcription factor NF-κB is

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<td>ELAM-1 ligand fucosyl transferase</td>
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<td>FGF-5</td>
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Table 2 Cumulative data on affected genes, the corresponding Genbank (GB) accession number and affected processes of cytokine-stimulated smooth muscle cells

up-regulated in activated cultured smooth muscle cells and encountered in the DD/RT-PCR analysis. As mentioned above, this observation with cultured smooth muscle cells coincides with a study showing that activated NF-κB is present in human atheromas, but not in apparently healthy arteries (36). The data are also in agreement with the finding that the products of many genes which are induced by NF-κB are detected in the plaque (49). NF-κB is also a crucial player in the delicate balance that determines apoptosis or protection against programmed cell death. These processes are of prime importance for the generation and regression of fully developed plaques. The properties of NF-κB to both promote apoptosis and to counteract this process are illustrated by the following findings. Activation of smooth muscle cells by a cocktail of cytokines (containing e.g. TNF-α) causes synthesis of NF-κB, as revealed by our DD/RT-PCR experiment (Table 2). As a consequence of the activation of the NF-κB pathway, down-stream genes are induced which are known to inhibit apoptosis, notably inhibitor of apoptosis protein-1 (hIAP-1) and FLICE-like inhibitory protein (FLIP) (50-54).

Next to the induction of NF-κB and its subsequent down-stream genes, it is also of interest to discuss the induction of the peroxisome proliferator-activated receptors (PPARs) in these cells and the probable mechanistic relation between the PPARs and NF-κB (55). The PPARs are transcription factors and members of the family of the nuclear steroid/thyroid hormone receptors. PPARα stimulates the β-oxidative degradation of fatty acids, whereas PPARγ regulates adipocyte differentiation and glucose homeostasis. PPARα has been linked to the NF-κB signal transduction pathway, since it antagonizes the nuclear translocation of NF-κB (56). It is conceivable that inhibition is mediated by the formation of inactive complexes between NF-κB and PPARα. These observations are consistent with the reported anti-inflammatory activities exerted by PPARα ligands (57, 58). Similarly, it was demonstrated that PPARγ ligands inhibit the development of atherosclerosis in LDL-receptor deficient mice (59). However, the explanation of this beneficial effect does not imply interference in the NF-κB pathway. Rather, it has been suggested that PPARγ ligands would reduce the expression of MCP-1 in macrophages and vascular-cell adhesion molecule-1 (VCAM-1) in endothelial cells (60-62), although some investigators did not observe these effects (59). Recently, different laboratories have reported exciting new data on the effect of activated PPARγ on lipid influx and efflux of macrophages and these results will be discussed in the following paragraph.

Interestingly, a distinct subfamily of the nuclear steroid/thyroid hormone receptor superfamily was found in our DD/RT-PCR of resting versus activated smooth muscle cells. This nerve growth factor inducible gene B (NGFI-B) subfamily consists of three genes which encode orphan nuclear receptors and are denoted TR3, mitogen-induced orphan receptor (MINOR) and nuclear orphan receptor of T cells (NOT) [reviewed in (63)]. All three members are induced in cultured smooth muscle cells, treated with an atherosclerotic stimulus. The NGFI-B family members have been implicated in different cellular processes and are also expressed by other vascular cells in atherosclerosis (E. K. Arkenbout, H. Pannekoek, C. J. M. de Vries,
unpublished observations). Presently, no data are available on the function of these receptors in atherogenesis.

Finally, we will mention new data which may serve as an example for the intricate regulation of gene expression which determines the phenotype of smooth muscle cells (15). In addition, it may be instructive to illustrate the potentially crucial role of the Sp/XKLF family of zinc finger transcription factors in atherogenesis. As stated before, dedifferentiation of quiescent, contractile smooth muscle cells into proliferative cells is characterized by down-regulation of contractile proteins, such as SM-actin and SM22α (13). Conversely, maintenance of the quiescent, contractile state is strongly promoted by transforming growth factor β (TGF-β) (1,2). This effect of TGF-β is mediated by a specific TGF-β control element (TCE), identified in the promoter of SM-actin and SM22α (15, 64). It was shown that members of the Sp/XKLF family of transcription factors interact with the TCE. Gut-enriched Krüppel-like factor (GKLF) was found to bind to the TCE and to repress TGF-β-promoted transcription of SM22α. Accordingly, expression of GKLF in proliferative smooth muscle cells is high and is specifically down-regulated by TGF-β when the cells become quiescent. In contrast to the repressing effect of GKLF, overexpression of another XKLF family member (BTEB2) augmented the TGF-β-dependent increase in SM22α mRNA synthesis: these findings are specific for BTEB2 since LKLF did not affect SM22α expression (15). In conclusion, the often ubiquitously expressed members of the Sp/XKLF family are structurally similar proteins that bind to the so-called GC/GT boxes in transcription regulatory elements. Nevertheless, they exhibit a high degree of specificity which is explained by a competitive mechanism in which differences between DNA binding affinity to a given GC/GT box and the relative concentration of the XKLFs would determine the prevalence of a particular family member (46). In aggregate, these studies together with the discussed effect of endothelial LKLF on the organization of smooth muscle cells during mouse embryogenesis, and possibly also post-natally, emphasize the importance of the Sp/XKLF family in the maintenance of the integrity of the vessel wall.

Monocytes/Macrophages

The attachment of monocytes to activated endothelial cells, followed by extravasation into the intima, are crucial steps in the development of atherosclerotic lesions. Monocytes differentiate in the vessel wall into macrophages and generate lipid-laden “foam cells”. To define the molecular mechanisms of gene expression during the process of macrophage differentiation, Hashimoto and colleagues employed SAGE to delineate differential gene expression upon the transition of monocytes into macrophages, induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) (65). Interestingly, in view of our specific identification of the involvement of the Sp/XKLF family of transcription factors in atherogenesis, these investigators detected down-regulation of a member of the XKLF family, although they did not unveil its explicit identity. Remarkably, none of the PPAR family members were identified in this screen. This result is unexpected since it has been shown that treatment of monocytes with ox-LDL causes differentiation into macrophages and induction of PPARγ expression (66,
The ultimate influence of PPARγ expression and activation on atherosclerosis has been puzzling, although recent observations have shed light on this enigmatic issue (see below). First, it has been shown that PPARγ-specific ligands inhibit the expression of inflammatory genes [e.g. TNF-α, interleukin-1β (IL-1β)] (68, 69). Hence, these results would indicate that PPARγ and its specific ligands act as anti-inflammatory and anti-atherogenic agents. Second, it has been demonstrated that activated PPARγ up-regulates the synthesis of CD36, a major scavenger receptor on macrophages which mediates uptake of ox-LDL and, consequently, promotes removal of its own inducer (i.e. ox-LDL) (66). It should be noted, however, that up-regulation of CD36 would be expected to be pro-atherogenic, since CD36 deficiency in mice susceptible to diet-induced atherosclerosis due to apoE deficiency (CD36-/--apoE-/-) protects against atherosclerosis (70). An apparently contradictory observation is that the PPARγ-specific agonists, rosiglitazone and GW7845, strongly inhibit the development of atherosclerosis in LDL receptor-deficient (LDL-R-/-) mice (59). These conflicting data might be reconciled by yet another effect of activated PPARγ. Notably, PPARγ ligands promote apoptosis by inhibiting the anti-apoptotic NF-κB signalling pathway (71). Possibly, programmed death and subsequent removal of macrophages may restrict the size of the lesion. Finally, recently, three “back-to-back” papers provided a rationale for the puzzling effect of PPARγ activation on atherosclerosis (72-74). It was demonstrated that the induction of CD36 by PPARγ is actually counteracted by a reduced expression of another scavenger receptor (SR-A). In addition, a nuclear receptor of the steroid/thyroid family LXR-α is up-regulated by PPARγ and induces the expression of the reverse cholesterol transporter ABCA1. Hence, the net result of PPARγ activation is anti-atherogenic in foam cells and now provides a rational explanation for the beneficial effects of PPARγ specific agonists.
Reference


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