Differential Display Identification of 40 Genes with Altered Expression in Activated Human Smooth Muscle Cells

LOCAL EXPRESSION IN ATHEROSCLEROTIC LESIONS OF smags, SMOOTH MUSCLE ACTIVATION-SPECIFIC GENES*

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Detailed knowledge on the molecular and cellular mechanisms that control (re)-differentiation of vascular smooth muscle cells (SMCs) is critical to understanding the pathological processes underlying atherogenesis. We identified by differential display/reverse transcriptase-polymerase chain reaction 40 genes with altered expression in cultured SMCs upon stimulation with the conditioned medium of activated macrophages. This set of genes comprises 10 known genes and 30 novel genes, which we call “smags” (for smooth muscle activation-specific genes). To determine the in vivo significance of these (novel) genes in atherogenesis, we performed in situ hybridization experiments on vascular tissue. Specifically, FLICE (Fas-associated death domain-like interleukin-1β-converting enzyme)-like inhibitory protein (FLIP) is expressed in neointimal SMCs as well as in lesion macrophages and endothelial cells, whereas the expression of the novel genes smag-63, smag-64, and smag-84 is restricted to neointimal SMCs. Characterization of full-length smag-64 cDNA revealed that it encodes a novel protein of 66 amino acids. smag-82 cDNA comprises the complete, unknown, 3'-untranslated region of fibroblast growth factor-5. Collectively, our results illustrate the complex changes of SMC gene expression that occur in response to stimulation with cytokines and growth factors secreted by activated macrophages. Moreover, we identified interesting candidate genes that may play a role in the differentiation of SMCs during atherogenesis.

The arterial vessel wall is composed of a single, luminal layer of endothelial cells, multiple layers of smooth muscle cells (SMCs), forming the media, and the adventitia. The principal function of medial SMCs is to provide the artery with elastic properties to maintain vascular tone in response to environmental stimuli. Therefore, SMCs are equipped with a contractile apparatus, involving cytoskeletal proteins, ion channels, and specific signaling molecules. Limited information is available on SMC-specific proteins except for those, which have been proposed to be associated with the cytoskeleton, notably SM α-actin, myosin heavy chain subtypes, desmin, vimentin, calponin, caldesmon, SM22α (reviewed in Ref. 1), and smoothelin, a recently described protein (2). A typical characteristic of vascular SMCs is that they easily undergo phenotypic changes, involving the transient transformation from resting, contractile, fully differentiated SMCs into proliferative, migratory, dedifferentiated SMCs, which synthesize vast amounts of extracellular matrix components (1, 3). This plasticity of SMC phenotype is relevant in adult organisms for maintenance of an intact vascular system upon vascular injury as well as during angiogenesis. In addition, accelerated proliferation and migration of SMCs plays an important role in the formation of atherosclerotic lesions, post-angioplastic restenosis, and vessel-wall extension in response to hypertension.

During atherosclerosis, activated endothelial cells allow massive infiltration of monocytes into the subendothelial space, where these cells differentiate into macrophages and are ultimately converted into lipid-laden foam cells (reviewed in Ref. 4). The continuous presence of macrophages in the vessel wall results in constitutive, local secretion of cytokines and growth factors. These, mostly unknown, factors affect the quiescent medial SMCs, which subsequently undergo a transition into migrating, proliferative SMCs. At present, limited information is available on the molecular mechanisms that control the (de)differentiation program of human SMCs and, more specifically, on the genes which are involved in these processes. It should be emphasized that the human genome has been estimated to comprise up to 100,000 genes and that a function has been assigned to only 5–10% of these genes. Therefore, we assume that most of the genes, which are involved in a multifactorial phenomenon like SMC phenotype transition, are unknown. An inventory of those (novel) genes will substantially improve our knowledge on the underlying mechanisms in SMC biology and pathology, and may allow the future identification of new targets for drug discovery. We cultured human SMCs in vitro and treated the cells with a stimulus that mimics the onset of atherogenesis when the initial lipid-laden macrophages have accumulated in the subendothelium. Differential

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§ The abbreviations used are: SMC, smooth muscle cells; SM, smooth muscle; RT-PCR, reverse transcriptase-polymerase chain reaction; DD, differential display; LDL, low density lipoprotein; IL, interleukin; kb, kilobase(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; nt, nucleotide; bp, base pairs; EST, expressed sequence tag; GM-CSF, granulocyte-macrophage colony stimulating factor; FGF, fibroblast growth factor; ORF, open reading frame; ICAM-1, intracellular adhesion molecule 1; FLICE, Fas-associated death domain-like interleukin-1β-converting enzyme; FLIP, FLICE-like inhibiting protein.
gene expression of SMCs was analyzed by differential display/RT-PCR (DD/RT-PCR) (5, 6).

In this report, we describe the identification of 40 (partial) cDNAs corresponding to mRNAs with regulated expression in activated SMCs. Among these genes are four known genes, which have been described to be involved in atherosclerosis, a set of six known genes, which have been described in relation to different cellular processes and 30 novel genes of unknown function, the so-called "smooth muscle activation-specific genes (smags)." The subsequent documentation of differential expression of these genes in vascular specimen of normal vascular tissue and at different stages of the disease and characterization of the corresponding full-length cDNAs allows us to propose a potential function of these (novel) genes in atherogenesis.

EXPERIMENTAL PROCEDURES

Human Tissue Samples—Arteries were dissected from human umbilical cords and immediately frozen or used for SMC explant cultures. Apparently normal vascular tissue and early stages of atherosclerosis were obtained during organ transplantation, whereas final stages of atherosclerotic vascular tissue was acquired during vascular surgery. In each case, informed consent was obtained of patients and/or relatives according to protocols of the Medical Ethical Committee of the Academic Medical Center of Amsterdam. Tissue samples for in situ hybridization and immunohistochemistry were fixed within 15 min after resection in 3.8% (v/v) formaldehyde in phosphate-buffered saline and were subsequently paraffin-embedded.

Tissue Culture—SMCs were obtained from human artery explant cultures (passage 5 to 7) and maintained in 1:1 mixture of RPMI and M199 (Life Technologies, Inc., Gaithersburg, MD) with 20% (v/v) human serum, penicillin, streptomycin, and fungizone. The cultured cells were characterized by immunofluorescence with a monoclonal antibody directed against SM a-actin (1A4, Dako, Denmark), which was detected with a goat-anti-mouse antibody (Jackson Laboratories, Westgrove, PA). With this method, the cells show uniform fibrillar staining. Confluent cultures were made quiescent by incubation overnight in serum-free medium, supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 μg/ml), and vitamin C (0.2 mM).

Oxidation of Human Low Density Lipoprotein (LDL) and Production of Conditioned Medium of Macrophages—LDL (200 μg of protein/ml) (Sigma, St. Louis, MO) was oxidized against phospholipase A2 and subsequently incubated for 7 h at 37 °C with copper sulfate (10 μM). The reaction was stopped by the addition of 100 μM EDTA. The oxidized LDL contained 40–50 nmol of thiobarbituric acid-reactive substances per mg of protein (7). Human elutriated monocytes (kindly provided by Dr. C. Shanahan (Cambridge, United Kingdom) (9) and to Dr. E. Meul, CLB, Amsterdam, The Netherlands) were allowed to attach to 80-cm² tissue culture flasks in 10 ml of RPMI/M199 with 5% human serum, penicillin, streptomycin, and fungizone. The cultured cells were harvested, stored at −20 °C, and were assayed, the latter ones as a control for specificity of the signal.

RNA Isolation and Northern Blotting—Total RNA was isolated with the TRIzol™ method (Life Technologies, Inc., Gaithersburg, MD) and was subsequently incubated for 7 h at 37 °C with copper sulfate (10 μM). The reaction was stopped by the addition of 100 μM EDTA. The oxidized LDL contained 40–50 nmol of thiobarbituric acid-reactive substances per mg of protein (7). Human elutriated monocytes (kindly provided by Dr. C. Shanahan (Cambridge, United Kingdom) (9) and to Dr. E. Meul, CLB, Amsterdam, The Netherlands) were allowed to attach to 80-cm² tissue culture flasks in 10 ml of RPMI/M199 with 5% human serum, penicillin, streptomycin, and fungizone. The cultured cells were harvested, stored at −20 °C, and were assayed, the latter ones as a control for specificity of the signal.

RESULTS

Characterization of Primary SMC Cultures

Although a substantial cellular part of the human atherosclerotic vessel wall consists of SMCs, the complexity and variability of these lesions, with respect to differences in cellular composition and differentiation status of those vascular cells, is too extensive to allow a direct comparison of gene expression...
patterns in normal and atherosclerotic tissues. Consequently, we applied in vitro cultured SMCs in our search for novel atherosclerotic, SMC-specific genes. At present, no stable human SMC line is available, which can either display a quiescent or a proliferative phenotype, like primary cells. SMCs were obtained from explant cultures of umbilical cord artery, adult iliac artery, or abdominal aorta and grown to confluency. Subsequently, we compared the relative expression levels of the SMC-specific differentiation markers SM22α, SM α-actin, and calponin in these cultures by Northern blotting (Fig. 1). Each of these genes exhibits the highest expression level in normal vascular tissue (lane 1), which is predominantly composed of fully differentiated, medial SMCs. SM22α is expressed in all the SMC cultures, originating from different vascular sources (panel A), with a relatively high expression level in umbilical cord artery SMCs. However, SM α-actin (panel B) as well as calponin (panel C) mRNA expression is restricted to the in vitro cultured SMCs obtained from umbilical cord artery. From these data we conclude that SMCs derived from this human, neonatal vascular tissue most closely resemble medial SMCs and are likely to exhibit the largest differences in gene expression upon activation, which makes them most appropriate for our studies.

Differential Display/RT-PCR Analysis on Stimulated SMCs

To mimic the conditions to which vascular SMCs are exposed at the initiation of atherosclerosis, we stimulated cultured, umbilical cord artery SMCs with the conditioned medium of human macrophages, which were activated with oxidized LDL particles. Confluent SMC cultures were kept overnight in serum-free medium and were subsequently stimulated for different periods with medium containing 5% (v/v) human serum and appropriately diluted conditioned medium of human macrophages. To discriminate between genes, which respond to serum and genes that are regulated by macrophage-secreted components, an additional stimulation was performed for a few selected periods with 5% (v/v) human serum only. To identify immediate early, early as well as late response genes, total RNA was isolated from SMCs after 0, 1, 2, 4, 8, and 24 h of stimulation. The incubations were performed in duplicate in separate experiments. For each RNA preparation, different cDNA fractions were generated with 12 two-base anchored oligo(dT)11, oligonucleotides (5). These cDNA fractions were amplified with 12 different random decamers (6) and the reaction products were analyzed on denaturing gels. Differential cDNA fragments that are present at identical periods of activation in both sets of RNA, were isolated from the gel, reamplified, and subcloned. Subsequently, the sequence of the differential display fragments was determined. Finally, these partial cDNA sequences were analyzed by means of the nonredundant GenBank/EMBL database to identify known genes with established functions. Homology searches against expressed sequence tag (EST) data bases and the High-Throughput Genome sequence data bases identified known sequences to which no function has been assigned yet and the novel sequences were deposited in GenBank (Table I). Representative examples of DD/RT-PCR reactions run on denaturing gels are shown in Fig. 2. The nucleotide sequence of the band

| Genbank accession number | Homology from-to (bp) |
|-------------------------|----------------------|
| Cellular interactions   |                      |
| ICAM-1                  | X06990               |
| ELAM-1                  | M58596               |
| Fuc-TIV                 |                      |
| SM α-actin              |                      |
| SM22α                   |                      |
| calponin                |                      |
| EthBr                   |                      |
| Signaling/gene activation|                      |
| GM-CSF                  | M11220               |
| Interleukin-8           | M28130               |
| PGF-5                   | M37929, AF171928     |
| NF-κB                   | M58693               |
| TR3 orphan receptor     | L13740               |
| Mitogen induced orphan receptor | U12767 |
| Inhibition of apoptosis  |                      |
| hIAP-1                  | AF070674             |
| FLICE-like inhibitory protein | U97075 |

Unassigned

| smag-9                  | AL049305.1           |
| smag-16                 | AL136447             |
| smag-20                 | AW129983             |
| smag-30                 | AL049694.9           |
| smag-37                 | AW566138             |
| smag-38                 | AW566139             |
| smag-40                 | AW566140             |
| smag-42                 | AW566141             |
| smag-50                 | AW566142             |
| smag-53                 | AC007319             |
| smag-54                 | AL138722             |
| smag-56                 | NT8142               |
| smag-58                 | AW566143             |
| smag-59                 | AW566144             |
| smag-60                 | AC020649             |
| smag-63                 | AW566145             |
| smag-64                 | AP707092             |
| smag-66                 | AC072226             |
| smag-71                 | AW566146             |
| smag-75                 | AC017002             |
| smag-83                 | AC012584             |
| smag-84                 | W42659               |
| smag-85                 | AF062223             |
| smag-86                 | AC018986             |
| smag-92                 | AW566329             |
| smag-95                 | AW566330             |
| smag-96                 | AW566331             |
| smag-97                 | AW566332             |
| smag-98                 | AW566333             |
| smag-99                 | AW566334             |

a GenBank accession number for obtaining full name, sequence, and references to published reports.

b The exact location of the homology of the sequences identified by DD/RT-PCR within the published sequence is given according to the numbering of the GenBank sequence.
Identification of genes with regulated expression in activated SMCs by DD/RT-PCR analysis. Resting SMCs (0 h) were stimulated for 1, 2, 4, 8, and 24 h with medium containing 5% (v/v) human serum and macrophage-conditioned medium, or for 6 and 24 h with medium containing 5% (v/v) human serum only (lanes 6 and 24). The stimulation was performed in duplicate in separate experiments with different batches of macrophage-conditioned medium (left and right part of this figure) and total RNA was isolated after the indicated periods. DD/RT-PCR reactions were performed with two base-anchored oligo(dT)11 and different decamers (6) and several typical examples are shown; T11GC and decamer 7 (A), T11CG and decamer 3 (B), T11CA and decamer 3 (C), T11GC and decamer 6 (D), T11GC and decamer 5 (E). The reactions were run on denaturing gels and parts of the corresponding autoradiograms are shown. The arrows indicate the bands, which are differentially expressed upon stimulation of the SMCs and show similar kinetics of expression in both sets of RNA. The bands were recovered from the gel, cloned, and sequenced and identified as GM-CSF (13), FLIP (14), smag-63 exemplifies a gene, which is induced only after 2 h of stimulation and are shut-off after 4 h. The expression of GM-CSF is transient from 2 to 8 h (see also Fig. 2), whereas interleukin-8 (IL-8) (17), ICAM-1 (18), and nuclear factor-kB (NF-kB) (19) are expressed throughout the stimulation period with optimal expression at 4 to 8 h. FLIP, ICAM, smag-53, and smag-99 are induced by 5% (v/v) human serum both in the presence and absence (lane 6c) of the macrophage conditioned medium, suggesting that these genes are at least partially induced by serum. In Table II, top, we summarized the extent of induction of the genes shown in Fig. 3A as determined by PhosphorImager analysis, as well as the size of the corresponding mRNA, deduced from the relative migration in the gel. Only 12 of the 40 partial cDNAs, isolated from the DD/RT-PCR analysis, reveal a hybridization signal on the total RNA blots, whereas the expression of the other mRNAs corresponding with the available probes is too low to be detected with this technique.

To confirm the expression patterns of the genes with relatively low expression levels, we performed RNase protection assays. In the RNase protection experiments, hybridizations were performed simultaneously with both a radiolabeled GAPDH-riboprobe and the riboprobe of the gene of interest. The GAPDH-protected band demonstrates equal loading of the lanes (example given in the last panel of Fig. 3B). In the first lanes the results are shown of a control hybridization of the riboprobes with tRNA and subsequent RNase digestion, which shows that the probes are not protected against RNase upon hybridization with nonspecific RNA. In Fig. 3B, a summary of the data obtained in the RNase protection assays is given, showing only the protected bands for each of the probes tested. From our initial experiments, we learned that the expression patterns observed in the DD/RT-PCR gels are similar in the Northern blots, an observation that allowed us to assay in the RNase protection analyses only the most informative periods of activation. For example, the RNase protection data for smag-63 exactly correspond with the DD/RT-PCR data shown in Fig. 2E.

Differential expression patterns were furthermore, confirmed for ELAM-1- and ICAM-1-genes as well as for other genes (23). As a first step to determine if any of the differentially expressed genes might play a role in atherogenesis, we analyzed the expression of a selected number of these genes in human vascular tissue by in situ hybridization. We determined the mRNA expression of FLIP and FGF-5, as well as of smag-84, which is represented in the EST data base, and of the genuine novel genes smag-63 and smag-64 in vascular specimens at different stages of the disease. The vascular specimens, which were applied in these experiments, were obtained from organ
Differential Gene Expression in Activated Human SMCs

Fig. 3. A, confirmation by Northern blotting analysis of regulated expression of mRNAs identified in the DD/RT-PCR analysis. Northern blots were prepared of total RNA isolated from (stimulated) SMCs and probed with partial cDNAs identified in the DD/RT-PCR analysis and the autoradiographs are shown. Resting SMCs (0 h) were stimulated for 1, 2, 4, 8, and 24 h with medium containing 5% (v/v) human serum and macrophage-conditioned medium, or for 6 and 24 h with medium containing 5% (v/v) human serum only (lanes 6' and 24'). From the top to the bottom, the results obtained with the following probes are shown: GM-CSF, IL-8, ICAM-1, NF-κB, TR3 orphan receptor, MINOR, FLIP, hIAP-1, smag-53, smag-64, smag-84, smag-99, and GAPDH as a control for equal loading. The relative intensity of the bands was determined by PhosphorImager, analyzed with ImageQuant software, and the data are summarized in Table II, top, in which also the length of the differentially expressed mRNAs is given. B, RNase protection assays to confirm differential expression of mRNAs identified in the DD/RT-PCR analysis. In this figure only the protected fragments of the RNase protection assays are given. In the first lane (tRNA control) it is shown that no aspecific hybridization is observed for each of the antisense riboprobes tested. The other lanes are labeled as in A. The blank lanes indicate that the corresponding time point was not assayed for that specific probe. For each differential display riboprobe, a simultaneous hybridization was performed with the GAPDH riboprobe as a control for the total amount of RNA applied in the experiment. In the last panel an example is given is the protected GAPDH band for a complete set of RNA samples. The relative intensity of the bands was determined by PhosphorImager, analyzed with ImageQuant software, and the data are summarized in Table II, bottom.

Identification of smag-82 as FGF-5—Fragments obtained from the DD/RT-PCR analysis comprise up to 500 bp and are usually derived from the 3’-untranslated region. Only 2 of the 30 novel genes display homology to EST sequences that are assembled in databases either at NCBI or at the Institute for Genomic Research (TIGR) (Table I). Obviously, full-length cDNAs would be better suited to predict a potential function of the encoded gene product. Accordingly, we constructed a cDNA library using a mixture of mRNAs isolated from both quiescent SMCs and from SMCs that were stimulated for different periods. The differential display fragment of smag-82 was used to screen pools of this (activated) SMC cDNA library, which resulted in the isolation of two independent cDNA clones of 5025 (82H) and 3535 bp (82F), respectively. Sequence analysis of these clones revealed, first, that the cDNA of 3535 bp is identical to the reported cDNA sequence of the short variant (82H) and 3535 bp (82F), respectively. Sequence analysis of these clones revealed, first, that the cDNA of 3535 bp is identical to the reported cDNA sequence of the short variant of FGF-5, which lacks the second exon (23). Until now only a partial cDNA of FGF-5 has been described (GenBank accession number M37825, 1123 bp), which encodes only the open reading frame (ORF) of human FGF-5. We now demonstrate that donors who did not have a prior history of vascular disease or from patients during reconstructive vascular surgery. Immunohistochemical analyses were performed on consecutive sections of these vascular specimens to assess the cellular composition of the specimens, in terms of the presence of neointimal SMCs and macrophages. An example of vascular tissue with a neointima of only a limited number of SMC layers is shown in Fig. 4, A–E. In this neointima macrophages are absent (Fig. 4B). FLIP mRNA is highly expressed in the endothelial cell lining of the normal vessel wall (Fig. 4C), in the adventitial capillaries (data not shown), and in some neointimal SMCs, whereas smag-84 and smag-64 are not expressed in this specimen. In an early lesion, containing both neointimal SMCs (Fig. 4, F and G) and lipid-laden macrophages (Fig. 4H), we studied the expression of these genes and found that FLIP is expressed both in neointimal SMCs and macrophages (Fig. 4I). However, smag-84 and smag-64 are not expressed in the macrophage foam cells as is shown in Fig. 4, J and K, respectively. The expression of smag-84 is restricted to some neointimal SMCs in this lesion (arrows in Fig. 4J), whereas no expression of smag-64 was observed in this lesion. Fig. 4, L–O, illustrate that smag-64 is expressed in an advanced aortic lesion by a subset of neointimal SMCs, which are scattered throughout the lesion. The expression pattern of smag-63 shows high similarity to the expression of smag-64 (data not shown). Finally, the level of expression of smag-82 (which represents a FGF-5 splice variant, see the following paragraph) is too low in these specimens to be detected by in situ hybridization (data not shown).
the complete FGF-5 transcript extends 5174 nt, which is in accordance with the largest hybridizing band that we observed by Northern blotting analysis (data not shown). At present, the function of the relative long 4654 bp 3′-untranslated sequence of FGF-5 is unknown.

Characterization of Full-length smag-64—Screening of the SMC cDNA library with the differential display fragment of smag-64, revealed a full-length cDNA of 2828 bp (see scheme Fig. 5A). The differential display fragment is located between bp 1240 and 1680, indicative for initiation of first strand cDNA synthesis at an internal adenyl stretch. Moreover, sequence analysis of the differential display fragment revealed that this fragment was amplified at both ends with the GC-anchored oligo(dT) primer. The orientation of this fragment was established by RNase protection assays with sense and antisense riboprobes. Application of 5′ rapid amplification of cDNA ends (numbers) (6) that identified the gene by DD/RT-PCR as described in Fig. 1.

The Northern blots revealed information on the length of the mRNA. For some genes multiple hybridizing bands were observed. Estimated length of the mRNA is given in kb.

Quantification of the radioactive bands was performed using a PhosphorImager with Image Quant software.

Table II

| Northern blot protection | Primer paira | Kineticsb | kb 44 | Inductionc |
|--------------------------|-------------|-----------|------|------------|
| GM-CSF                   | CG 7        | 2–8       | 1.0  | 10         |
| IL-8                     | GT 3        | 1–24      | 1.8  | >200       |
| ICAM-1                   | GG 12       | 1–24      | 3.3  | 8          |
| NF-κB                    | GT 4        | 2–24      | 1.7  | 6          |
| TR3 orphan c             | CA 3        | 1–4       | 5.0  | >200       |
| MINOR                    | CC 2        | 1–4       | 4.8  | >60        |
| FLIP                     | CG 3        | 2–8       | 4.7–2.5–1.5 | 15 |
| hIAP-1                   | GC/CA/AG/AC 2 | 1–8      | 8.0–6.4–5.5 | 80 |
| smag-53                  | CC 6        | 4–24      | 11.0 | 6          |
| smag-83                  | CG GC       | 2–4       | 3.3  | 8          |
| smag-99                  | CA/AG 3     | 2–4       | 4.7–7.0 | 14 |

Rapid amplification Primer paira | Kineticsb | Inductionc |
|--------------------------|-----------|------------|
| h 44 | -fold |
| ELTF/Fuc-TIV              | CG 4       | 8          | 2    |
| smag-82/FGF5              | AG 2       | 1–8        | 30   |
| smag-42                   | GA 5       | 2–8        | 4    |
| smag-50                   | CC 9       | t = 1 off  | t = 1 off | |
| smag-56                   | CT 2       | 1          | 20   |
| smag-60                   | CT 7       | 1          | 3    |
| smag-63                   | GC 5       | 4          | 5    |
| smag-66                   | GC 9       | 8          | 120  |
| smag-71                   | CA 2       | 1–4       | >200  |
| smag-75                   | GT 1       | 1–2       | 9    |
| smag-83                   | AG 2       | 1–4       | 11   |
| smag-85                   | AG 3       | 2          | 10   |

a Primer pair represents the combination of anchored primer (letters) and decamers (numbers) (6) that identified the gene by DD/RT-PCR as described in Fig. 1.
b Kinetics of (optimal) expression of the gene (in hours).
c The Northern blots revealed information on the length of the mRNA. For some genes multiple hybridizing bands were observed. Estimated length of the mRNA is given in kb.
d Quantification of the radioactive bands was performed using a PhosphorImager with Image Quant software.

DISCUSSION

We performed extensive DD/RT-PCR analysis on cultured human SMCs stimulated with conditioned media from activated macrophages as a means to identify potential candidate genes involved in mediation of alterations in SMC differentiation and growth associated with atherogenesis. The assay was performed on RNA samples isolated from SMCs, which were stimulated for different periods in two independent experiments, to achieve optimal efficacy and reproducibility of the DD/RT-PCR analysis. Only bands with a regulated expression in both experiments, displaying reproducible kinetics of mRNA expression were considered for further characterization. We applied 144 different primer combinations in our DD/RT-PCR analysis (see "Experimental Procedures"), which should represent, based on statistical calculations, 80% of the entire repertoire of mRNAs (6). Collectively, we identified 10 known and 30 novel genes with modulated expression in stimulated SMCs. Our analysis substantially extends the list of genes that are potentially involved in SMC de-differentiation during atherogenesis. Furthermore, our data indicate that multiple functions of SMCs are affected by the in vitro stimulus chosen (see Table 1). With regard to the differentially expressed known genes, the cellular adhesion molecule ICAM-1 has been reported to be induced in in vitro stimulated human SMCs (25), in addition to its induction in endothelial cells. Moreover, ICAM-1 is expressed in neointimal SMCs in the atherosclerotic lesion (26, 27). Induction of GM-CSF expression in cultured SMCs has been well documented (4, 28), whereas expression in the atherosclerotic vessel wall has only been reported in rabbits (29).
We and others detected a strong induction of IL-8 expression in activated SMCs (30), although so far, IL-8 expression had been only reported in endothelial cells and macrophages in the plaque (31, 32). As IL-8 has been shown to be a mitogen and chemoattractant for cultured SMCs, it may be speculated that autocrine stimulation is involved in the pathologic behavior of SMCs in atherosclerosis (33). Fuc-TIV/ELAM-ligand fucosyltransferase is an intracellular enzyme mediating the biosynthesis of a complex carbohydrate group, the so-called Lewis X antigen, which forms an essential part of the ligands for selectins (21, 22). Until now, Fuc-TIV/ELAM-ligand fucosyltransferase expression has only been described in leukocytes and is involved in the regulation of leukocyte-endothelial cell interactions. The enhanced expression of this fucosyltransferase in SMCs may result in the expression of selectin ligands on the cell surface, potentially allowing SMC migration from the media into the neointimal space during atherogenesis. The conditioned medium of oxidized LDL-stimulated macrophages is known to contain a relatively high concentration of tumor necrosis factor α, which explains the induction of NF-κB, a key mediator of tumor necrosis factor α signals. Involvement of NF-κB in atherosclerosis has been substantiated by the detection of tumor necrosis factor α, which explains the induction of NF-κB, a key mediator of tumor necrosis factor α signals. Involvement of NF-κB in atherosclerosis has been substantiated by the detection of tumor necrosis factor α, which explains the induction of NF-κB, a key mediator of tumor necrosis factor α signals.
tion of this protein in the atherosclerotic lesion in neointimal SMCs, endothelial cells, and macrophages with specific antibodies that only recognize the activated form of NF-κB (34).

Recently, NF-κB has been shown to regulate an anti-apoptotic mechanism in SMCs that involves direct induction of the expression of hIAP-1 (35). We demonstrate here that hIAP-1 as well as FLIP, which both have been characterized as inhibitors of the intracellular caspases (14, 36), are induced upon stimulation of SMCs, exemplifying the defense strategy of activated SMCs against programmed cell death during atherogenesis.

Finally, we show a transient induction of two members of a subfamily of the steroid/thyroid hormone receptor superfamily, TR3 orphan receptor, and MINOR (15, 16). It has been reported that these transcription factors are activated in many different processes involving cellular activation, differentiation, as well as in apoptosis (37, 38).

We recently described a similar, extensive DD/RT-PCR analysis with the same set of primers on resting and activated endothelial cells and found 106 differentially expressed genes (10). Remarkably, a very limited overlap of identified differentially expressed genes between endothelial cells and SMCs was observed. Among the known genes, only GM-CSF, IL-8, and hIAP-1 were differentially expressed in both endothelial cells and SMCs, whereas none of the novel genes or the genes represented in the EST data bases were identified in both screens. Among the novel genes isolated from endothelial cells, 33 out of 84 were represented in the EST data bases, whereas for SMCs only 2 out of 30 novel genes are ESTs. The mere fact that the human cDNA libraries, which were applied to generate the EST data bases, were made from only a very limited number of vascular SMC-containing tissues and not from tissues containing activated SMCs, may explain the low representation of our *smags* in these data bases.

The physiological relevance of the genes, which were isolated...
from the cultured SMCs, in atherogenesis was substantiated by mRNA expression studies in human vascular tissue at different stages of the disease. We show that FLIP is expressed in endothelial cells of the normal vessel wall, which is in accordance with the observation that FLIP quiescent, in vitro cultured endothelial cells express FLIP (39). In the latter study, it was further shown that the oxidized LDL-mediated induction of apoptosis in endothelial cells is due to the fact that FLIP expression is repressed. So far, we did not observe a clear repression of FLIP mRNA expression in endothelial cells covering the atherosclerotic lesion. However, within the plaque FLIP is prominently expressed in subsets of neointimal SMCs as well as in lipid-laden macrophages, which may indicate that both these SMCs and macrophages are protected against apoptosis by overexpression of this caspase homologue. Clearly, neointimal SMCs that are in close vicinity within a lesion can be divided in distinct subpopulations, which are characterized by specific expression patterns of these (novel) genes, for example, smag-64 and smag-84 (Fig. 4).

Most importantly, this DD/RT-PCR analysis provided us with a set of partial cDNAs which have not been isolated before, and for which a potential role of the encoded proteins in the pathologic behavior of SMCs in atherosclerosis can be anticipated. The set of novel genes, now available for in situ hybridizations, will enable us to analyze heterogeneity of SMCs in normal vascular tissue and in atherosclerotic lesions in more detail. The in situ expression data presented in this study illustrate that also in advanced lesions, which have been formed during decades, differences in gene expression patterns exist between the neointimal SMCs. This may indicate that even at later stages of the disease dynamic processes are involved in further progression of the lesion. The identification of the corresponding full-length cDNAs of the differential display fragments will allow us to study the functional involvement of the corresponding gene products in atherogenesis and to better understand the underlying processes of this disease. Ultimately, these studies will encourage us to define new targets for therapy in atherosclerosis and restenosis in angioplasty.

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