Identification of syntenin and other TNF-inducible genes in human umbilical arterial endothelial cells by suppression subtractive hybridization

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Abstract Endothelial cells play an important regulatory role in inflammatory responses by upregulating various proinflammatory gene products including cytokines and adhesion molecules. A highly potent mediator of this process is tumor necrosis factor-α (TNF). In the present study, the suppression subtractive hybridization (SSH) method was employed to identify rarely transcribed TNF-inducible genes in human umbilical arterial endothelial cells. Following mRNA isolation of non-stimulated and TNF-stimulated cells, cDNAs of both populations were prepared and subtracted by suppression PCR. Sequencing of the enriched cDNAs identified 12 genes differentially expressed including vascular cell adhesion molecule-1, monocyte chemoattractant protein-1, interleukin-8 and IκBα, an inhibitor of the transcription factor nuclear factor-κB. Interestingly, also syntenin, a PDZ motif-containing protein which binds to the cytoplasmic domain of syndecans, was identified by SSH. Time course studies using RT-PCR analysis confirmed that all genes were differentially expressed and rapidly induced by TNF. Our data reveal that SSH is a powerful technique of high sensitivity for the detection of differential gene expression in primary arterial endothelial cells.

Key words: Adhesion molecule; Endothelial cell; Syntenin; Nuclear factor-κB; Suppression subtractive hybridization; Tumor necrosis factor

1. Introduction

Tumor necrosis factor-α (TNF) is a potent proinflammatory cytokine with a broad range of biological activities which are held responsible for the pathogenesis of various inflammatory disorders, such as septic shock, cardiovascular dysfunction and atherosclerosis. A primary cellular target of TNF is the vascular endothelium where it initiates inflammatory responses by enhancing adhesion molecule expression and cytokine secretion [1,2]. An important component in this process is the transcription factor nuclear factor-κB (NF-κB) which is rapidly activated following binding of TNF to the TNF-R1 receptor [3]. In unstimulated cells, NF-κB is found as a heteromeric complex which is retained in the cytoplasm by its inhibitory subunit IκBα. A key event in the activation pathway of NF-κB is the inducible phosphorylation of IκBα, followed by its conjunction to ubiquitin and the subsequent rapid degradation of IκBα at the proteasome [4–7]. This reaction allows the active form of NF-κB to translocate to the nucleus and to bind to regulatory sequences of target genes. Among important target genes of NF-κB in endothelial cells are those encoding inflammatory and chemotactic cytokines such as interleukin (IL)-1, IL-6, and IL-8, cell adhesion molecules such as E-selectin, ICAM-1, MadCAM-1 and VCAM-1, and proinflammatory enzymes such as inducible nitric oxide synthase and cyclooxygenase-2 [8,9]. The coordinated expression of these molecules by activated endothelial cells initiates the inflammatory cascade and leads to tight leukocyte adhesion followed by their transmigration into tissue spaces [10].

There is ample evidence that NF-κB and IκBα can interact in an autoregulatory mechanism. NF-κB itself activates transcription of the IκBα gene, which decreases NF-κB activation and expression of κB-dependent genes [11–14]. Once translocated to the nucleus, NF-κB may be additionally regulated and controlled by IκB-dependent processes [15]. Thus, in vitro IκBα is capable of specifically displacing NF-κB dimers from the κB-elements of the E-selectin and VCAM-1 genes, indicating a post-induction transcriptional repression of the adhesion molecules [16].

In addition to the well-studied endothelial adhesion molecules such as E-selectin and VCAM-1, the family of transmembrane heparan sulphate proteoglycans, named syndecans, has been implicated in cell-matrix and cell-cell adhesion in many cell types including endothelial cells [17]. Several cell adhesion molecules and extracellular matrix proteins bind heparan sulphate, thereby mediating cell-cell and cell-matrix interactions. The heparan sulphate mediated binding of extracellular ligands and growth factors plays a central role for the functional activity of syndecans [18,19]. Syndecans seem to be essential regulators of ligand-dependent activation of primary signalling receptors at the cell surface.

In an effort to identify TNF-inducible proinflammatory gene products in endothelial cells, in the present study a recently developed method, termed suppression subtractive hybridization (SSH) was used [20,21]. This method combines a

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Abbreviations: HUAEC, human umbilical arterial endothelial cells; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-κB; IκB, inhibitor of NF-κB; PCR, polymerase chain reaction; RT, reverse transcription; SSH, suppression subtractive hybridization; TNF, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1

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high subtraction efficiency with an equalized representation of differentially expressed sequences. It is based on a specific form of polymerase chain reaction (PCR) permitting exponential amplification of cDNAs which differ in abundance, whereas amplification of sequences of identical abundance in two populations is suppressed. Here, we describe the identification of TNF-inducible genes by SSH in human umbilical arterial endothelial cells.

2. Materials and methods

2.1. Isolation and characterization of human umbilical arterial endothelial cells (HUAEC)

Endothelial cells were isolated from human umbilical cord arteries as described [22,23] and cultivated on fibronectin-coated culture dishes in Medium 199 supplemented with 20% FCS, 10 mg/ml heparin and 30 μg/ml crude endothelial cell growth factor. The endothelial origin of the cells was confirmed by indirect immunoperoxidase staining for von Willebrand factor.

2.2. Isolation of poly(A)$^+$NA and cDNA synthesis

The SSH was performed between unstimulated HUAEC (driver) and HUAEC stimulated for 4 h with 50 ng/ml TNF-α (tester). For preparation of polyadenylated RNA, total RNA was extracted with the TRIzol reagent and purified with the Poly(A)Quick kit (Stratagene, Heidelberg, Germany). The further steps were performed according to the PCR-Select™cDNA Substraction kit (Clontech, Palo Alto, CA, USA). For the first-strand cDNA synthesis 1 μg of the tester- and driver-poly(A)$^+$RNA were incubated with oligo(dT) primer for 2 min at 70°C and rapidly chilled on ice. The reverse transcription reaction was performed in RT-buffer (50 mM Tris–HCl, 6 mM $MgCl_2$ and 75 mM KCl) with 1 mM dNTPs and 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase for 1.5 h at 42°C. After this reaction the second strand cDNA synthesis followed immediately by incubating the first strand cDNA for 2 h at 16°C in 100 mM KCl, 10 mM ammonium sulphate, 5 mM $MgCl_2$, 0.15 mM β-NAD, 20 mM Tris–HCl, 0.05 mg/ml BSA containing 1 mM dNTPs, 0.3 U/μl DNA polymerase I, 0.01 U/μl RNase H and 0.06 U/μl *Escherichia coli* DNA ligase. The double-stranded cDNA was blunted by T4 DNA polymerase for 30 min at 16°C. The reaction was stopped by the addition of EDTA/glycogen followed by the precipitation of the cDNA. To obtain shorter blunt-ended molecules the cDNA was digested with 15 U *Rsa*I at 37°C for 1.5 h.

2.3. Adapter ligation and subtractive hybridization

The adapter ligation was performed only with the reverse transcribed and digested mRNA of the tester cDNA. One half of the tester cDNA was ligated with 2 μM adapter-1, the other half with 2 μM adapter-2 in the ligation mixture (50 mM Tris–HCl pH 7.8, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 5% polyethylene glycol and 0.5 U T4 DNA ligase) at 16°C overnight. The reaction was stopped by EDTA/glycogen and the ligase was inactivated by heating the samples at 72°C for 5 min. For the first hybridization an excess of driver cDNA was added to each tester cDNA (ligated with adapter-1 or -2) in separate samples. After denaturation at 98°C for 1.5 min the first hybridization was performed in a hybridization buffer at 68°C for 8 h. For the second hybridization the two samples of the first hybridization were combined without denaturation at 68°C overnight.

2.4. PCR amplification

A primary PCR was used to selectively amplify the differentially expressed sequences and performed with 1 μl of the diluted subtracted cDNA in a 25 μl volume containing 400 nM of each primer, 0.2 mM dNTPs, and 0.5 μl of Advantage Klen*Taq* polymerase mix (Clontech). The PCRs were performed in a thermal cycler (Perkin Elmer 480) with the following parameters: 75°C for 7 min, 30 cycles at 94°C for 30 s, 68°C for 30 s and 72°C for 1.5 min. Then, 1 μl of the amplified product was used as a template in secondary PCR for 30 cycles with nested adapter-specific primers under the following conditions: 94°C for 30 s, 68°C for 30 s and 72°C for 1.5 min.

2.5. Detection, cloning and sequencing of subtracted cDNA fragments

Subtracted cDNA fragments were detected by horizontal polyacrylamide electrophoresis. 7 μl of the amplified cDNA were separated on a long-run gel in three slots. The gel was subsequently cut in the second lane and the first part was stained with silver. Only distinct visible bands were excised from the unstained part of the gel and used as a template for another PCR. The products of the reamplified cDNA fragments were then inserted into the pCR2.1 cloning vector (TOPO-TA Cloning-kit, Invitrogen, Carlsbad, CA, USA) and transformed in Top F10′ cells (Invitrogen). The bacteria were plated on ampicillin-containing agar plates, which were overlaid with 1.4 μg β-X-Gal and 100 μM IPTG. After overnight incubation white colonies were picked and transferred into wells of a multititer plate with 200 μl LB medium and subsequently incubated for 4 h at room temperature. The bacteria were lysed by heating to 100°C for 10 min and 5 μl of each sample were used to amplify the cloned inserts in 50 μl reactions using standard PCR conditions and M13 sequencing primers. An aliquot of the PCR products was loaded on a horizontal polyacrylamide gel to confirm the correct size of the cloned fragment. DNA sequencing was performed by automated means at MWG-Biotech (Ebersberg, Germany). Nucleic acid homology searches were performed using the BLAST program [24].

2.6. Reverse transcription (RT)-PCR of detected sequences

To confirm the induction and differential expression of the detected sequences, RT-PCR analyses were performed. 5′- and 3′-primers were designed for each of the detected sequences (Table 1) and synthesized by MWG-Biotech (Ebersberg, Germany). PCR conditions were optimized by varying the annealing temperature and time as well as the Mg^{2+} and K^+ concentrations in the PCR buffer. Total RNA (4 μg) of unstimulated HUAEC and HUAEC stimulated with 50 ng/ml TNF was incubated with MMLV reverse transcriptase (200 U/μl) in a 40 μl volume containing 1×RT buffer (50 mM Tris–HCl pH 8.3, 75 mM

Table 1
Location and sequences of the optimal primers

Primer	PCR fragment size (bp)	Sequence	Location on cDNA
5′-GAPDH	287	5′-GCCAAAAGGGTCATCATCTC-3′	406–425
3′-GAPDH	287	5′-GTAGAGGCAGGGATGATGTTC-3′	672–692
5′-VCAM-1	376	5′-CTATACCATCCGAAAGGCCC-3′	1786–1805
3′-VCAM-1	376	5′-CATTCAGGGAAGTCTGCCTC-3′	2141–2160
5′-IL-8	375	5′-CGGAAGGAACCATCTCACTG-3′	1554–1574
3′-IL-8	375	5′-CACTGGCATCTTCACTGATTC-3′	3412–3432
5′-MCP-1	265	5′-CAGCCAGATGCAATCAATGC-3′	123–142
3′-MCP-1	265	5′-GTTAGCTGCAGATTCTTGGG-3′	369–388
5′-IκBα	324	5′-GGTGTACTTATATCCACACTGC-3′	1143–1164
3′-IκBα	324	5′-GGAGGGTAACACAAACCTTG-3′	1448–1467
5′-Syntenin	388	5′-GCTTGAACTGTCGCCTTAAC-3′	1624–1643
3′-Syntenin	388	5′-GACCATCCCAAAGTAGCTAG-3′	1993–2012
5′-IL-6	310	5′-GACAGCCACTCACCTCTTCA-3′	181–200
3′-IL-6	310	5′-GTACTCATCTGCACAGCTC-3′	472–490
5′-IL-1β	468	5′-GCATCCAGCTACGAATCTCC-3′	226–245
5′-IL-1β	468	5′-GATCTACACTCTCCAGCTGTAG-3′	672–693

KCl, 3 mM $MgCl_2$), 10 mM DTT, 0.5 mM dNTP, 50 U RNA guard and 100 pmol random hexamer primers for 5 min at 25°C, 5 min at 30°C, 90 min at 37°C and 5 min at 95°C. 1 μl of the sample was then amplified in a 50 μl reaction containing 1×PCR buffer (Perkin-Elmer), 0.2 mM dNTP, 20 pmol of each primer and 2.5 U *Taq* polymerase under the following conditions: 5 min at 94°C; 25–30 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min; extension at 72°C for 20 min. 20 μl of each sample were loaded onto a 1.5% agarose gel and stained with ethidium bromide. In order to determine the exponential phase of the RT-PCR, amplifications were performed in the presence of 0.1 μCi [α-^{32}P]CTP. After 15, 20, 25, 30, 35 and 40 cycles samples were removed and loaded onto ethidium bromide stained agarose gels. The bands were cut out under UV-light and melted in 500 μl H_2O. The relative amounts of amplified DNA were determined by Cerenkov counting and plotted as a function of the number of cycles. Densitometric analyses were performed on a two-dimensional scanning densitometer (Biometra, Göttingen, Germany) using the ScanPack software version 14.1A27. The ethidium bromide-stained gels were photographed and the densitometric results of gene expression were standardized to that of GAPDH expression in the same sample according to Ko et al. [23]. GAPDH transcripts were amplified in 25 PCR cycles. The ratio of the relevant expression profile versus the GAPDH profile was set to 100%. Statistical analysis of data was performed using the Mann–Whitney U test [25].

3. Results

In an effort to identify TNF-inducible genes in primary endothelial cells, the mRNA of HUAEC stimulated with TNF-α (tester) and non-stimulated HUAEC (driver) was isolated, reverse transcribed into cDNA and digested with *Rsa*I. For the SSH procedure two different adapters were separately ligated to the digested tester cDNA. After the first hybridization of the two different tester cDNAs with an excess of denatured driver-cDNA in separate samples and a second hybridization step, the subsequent PCR amplification was performed without denaturation. Following polyacrylamide electrophoresis subtracted cDNA fragments of different size were detected (Fig. 1A). The visible subtracted cDNA fragments were excised from the gel and reamplified. As shown in Fig. 1B, discrete cDNA fragments could be resolved by this procedure. The reamplified cDNA fragments were inserted into a cloning vector and sequenced using M13 primers. Nucleic acid homology searches identified 15 different cDNAs from which 12 sequences represented differentially expressed genes of known function (Table 2).

The genes identified by SSH could be classified into two groups. Six of the genes encoded proteins generally involved in cell cycle regulation, growth regulation and protein synthesis, such as ubiquitin activating enzyme E1, cdc2 kinase, the cyclin-dependent kinase inhibitor p21, stress-activated protein kinase p54, elongation factor-1α, HS1 protein and DnaJ. The second group of genes were involved in the regulation of proinflammatory events such as endothelial cell adhesion and leukocyte activation. These cDNAs encoded vascular cell adhesion molecule-1 (VCAM-1), IL-8, monocyte chemoattractant protein-1 (MCP-1) and IκBα, an inhibitor of NF-κB. Interestingly, we also found that syntenin, a novel PDZ motif containing adapter protein, which has been shown

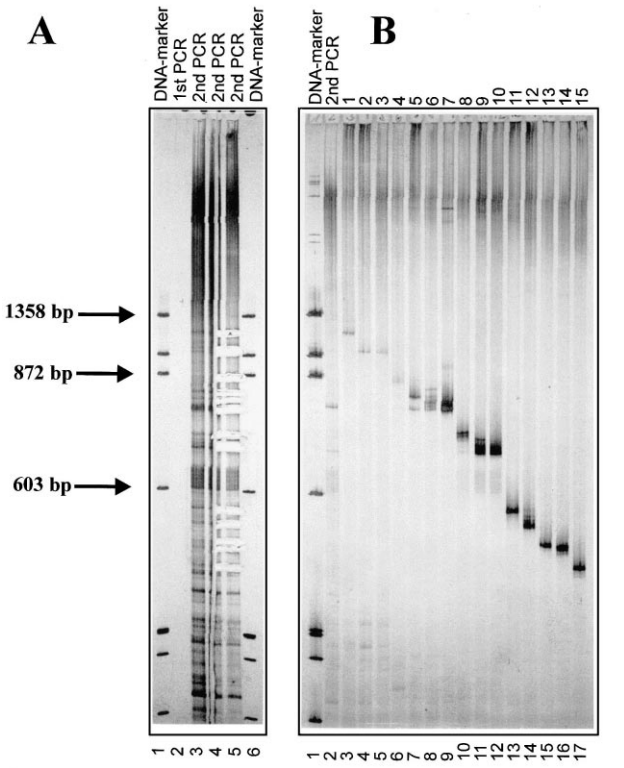

Fig. 1. Results of SSH. A: Reverse transcribed mRNA of HUAEC stimulated for 4 h with TNF was subtracted against reverse transcribed mRNA of non-stimulated HUAEC according to the SSH protocol. Lane 2 shows the PCR products of the first PCR and lanes 3–5 show the products of the second PCR. Distinct cDNA fragments were detected by polyacrylamide electrophoresis and excised from the gel (lane 5). Lanes 1 and 6: *Hae*III digest of ΦX-174 DNA. B: Result of the reamplifications of the cut subtracted cDNA fragments. The 15 single PCR products of the second PCR were reamplified and an aliquot of each sample was loaded on the gel (lanes 3–17). Lane 1: *Hae*III digest of ΦX-174 DNA, lane 2: PCR products of the second PCR.

Table 2
Differentially expressed genes of known function

Locus	Accession	Identified gene	Location (nt)
HSQAE1	X56976	ubiquitin activating enzyme	2562–2762
HSCDC2	X05360	cdc2 kinase	629–1049
HUMSDI1A	L26165	cyclin-dependent kinase inhibitor p21	1877–2027
HSY11298	Y11298	stress-activated protein kinase p54	922–1131
HSEF1AR	X03558	elongation factor-1α	415–835
HSHS1RNA	X57347	HS1 protein	1027–1507
HUMDNAJA	D13388	DnaJ	545–992
HUMVCAM1V	M30257	VCAM-1	1763–2310
HUMIL8A	M12830	IL-8	3556–4021
HSMCP1	X14768	MCP-1	122–528
HUMMAD3A	M69043	IκBα	1140–1481
AF000652	AF000652	syntenin	1515–1734

to bind to the cytoplasmic domains of syndecans and other transmembrane receptors [26,27], was stimulated by TNF.

To investigate whether the genes for syntenin and the other proinflammatory mediators were indeed inducible by TNF as suggested by the previous SSH data, we performed detailed semi-quantitative PCR analyses. In the first experiments the exponential amplification phases for these genes were determined by radioactively labeled RT-PCR (Fig. 2). Further amplifications were then performed using 30 PCR cycles for VCAM-1, IL-8, MCP-1, and IκBα, while syntenin mRNA was amplified in 26 cycles. As detected by RT-PCR, stimulation of HUAEC with TNF caused a time-dependent elevation of the transcripts specific for VCAM-1, IL-8, MCP-1, IκBα and syntenin (Fig. 3). In contrast, expression of GAPDH was not altered upon TNF stimulation, confirming that equal amounts of mRNA had been used. Densitometric analysis of the ethidium bromide-stained gels of three further independent experiments for each gene product revealed a 4–7-fold increase of mRNA expression in response to stimulation with TNF. The expression of VCAM-1, IL-8 and MCP-1 specific mRNAs showed a contineous increase with maximal expression levels after 4 h, whereas induction of IκBα and syntenin mRNA expression was biphasic reaching two peaks after 1 and 4 h of cell stimulation (Fig. 4).

In our experiments we noticed that the expression of IL-1β and IL-6 that are known to be inducible by TNF was not detected by SSH. We therefore investigated whether the failure to identify both genes was due to the SSH or caused by a lack of TNF inducibility in our cell system. Fig. 5 demonstrates that IL-6 was not induced by TNF in HUAEC, as amplification of RNA taken either from the SSH reaction or from independent time course experiments resulted in similar amounts of IL-6 specific products. In contrast, IL-1β transcripts were time-dependently induced by TNF, but presumably escaped SSH detection due a low induction ratio. Nonetheless, the results reveal that most proinflammatory genes identified by the SSH approach were inducible by TNF. Furthermore, we demonstrate that expression of syntenin, whose transcriptional regulation has not been studied so far, follows a kinetic similar to other TNF-inducible gene products, suggesting that it might presumably represent a NF-κB target.

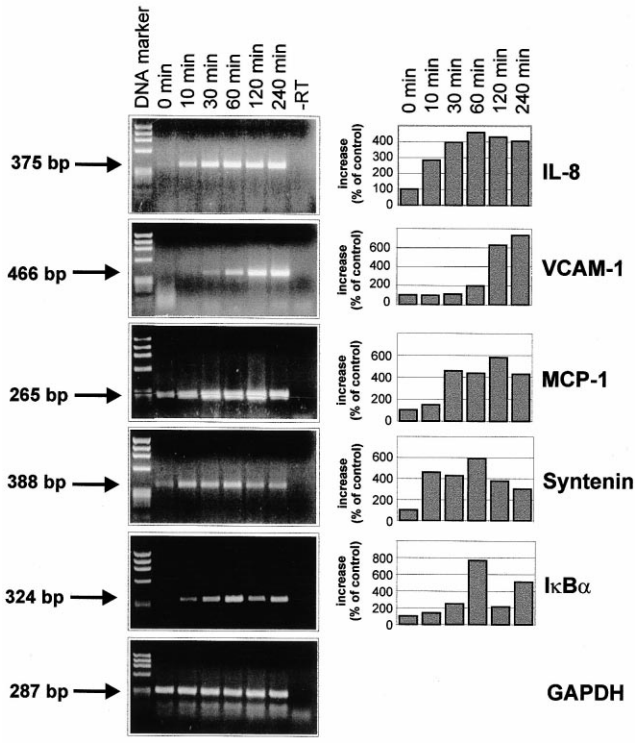

Fig. 3. Expression of IL-8, VCAM-1, MCP-1, syntenin and IκBα specific mRNA in response to TNF stimulation. HUAEC were incubated in medium alone or treated with 50 ng/ml TNF for the indicated times. Total RNA was isolated and RT-PCR was performed as described in Section 2. The molecular sizes of the resulting PCR products are shown on the left. As a control for the same amount of RNA a RT-PCR with GAPDH primers was performed (lower panel). The relative quantities of the specific PCR products were determined by densitometric analysis and normalized with the density of the GAPDH profiles (right panels).

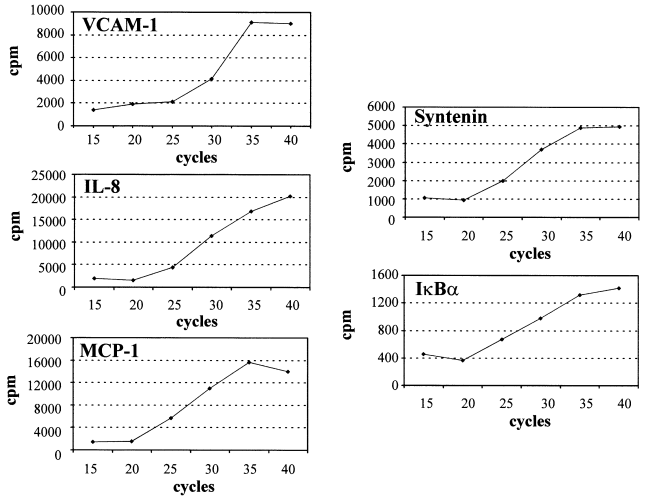

Fig. 2. Determination of the exponential phase of the RT-PCR. To avoid artificial results of the PCR, the amplification of the reversed transcribed RNA (4 μg total RNA) was performed for VCAM-1, IL-8, MCP-1, IκBα and syntenin with the constructed primers in the presence of 0.1 μCi [α-^{32}P]CTP. After 15, 20, 25, 30, 35 and 40 cycles samples were loaded on an ethidium bromide stained 1.5% agarose gel. Bands were cut out under UV-light and melted in 500 μl H_2O. The relative amounts of amplified DNA were determined by Cerenkov counting in a scintillation counter and plotted as a function of the number of cycles.

4. Discussion

The aim of the present study was to identify TNF-inducible genes in arterial endothelial cells by SSH, a novel equalizing cDNA subtraction method. TNF is a critical mediator of leukocyte adhesion to the vascular endothelium which constitutes a key step for the initiation of inflammatory responses and repair of tissue injury. At sites of inflammation endothelial cells develop a characteristic morphology and synthesize new proteins, an alteration termed endothelial activation [2]. Many inducible genes are involved in endothelial activation including VCAM-1, E-selectin, tissue factor, IL-1, IL-6, IL-8, G-CSF and others [8,9,16]. In this study, we identified 15 genes in activated endothelial cells, from which 80% were differentially expressed upon TNF stimulation. Seven of the genes were related to the regulation of cell growth, cell cycle and protein synthesis, whereas the five remaining genes, which

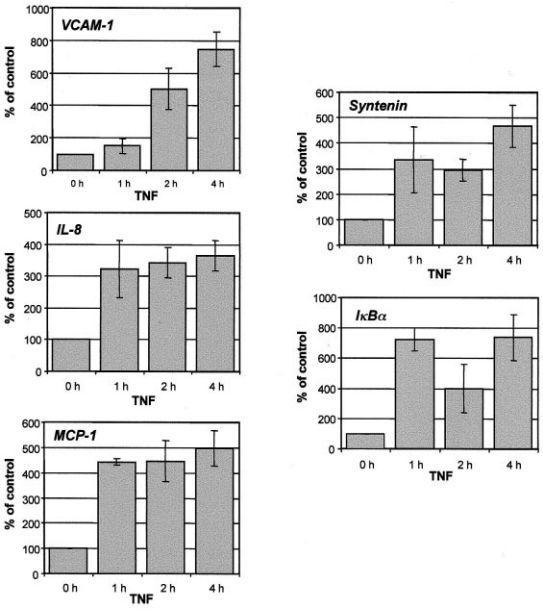

Fig. 4. Statistical analysis of VCAM-1, IL-8, MCP-1, IκBα and syntenin gene expression after TNF stimulation. The individual RT-PCR profiles from three independent experiments were subjected to densitometry and statistically analyzed with regard to GAPDH expression. Data of gene expression are presented as means ± S.D.

we have analyzed here in more detail, were involved in the control of inflammatory processes. In addition, by several time course studies using RT-PCR analyses we show that expression of these latter genes was indeed rapidly activated by TNF, which further demonstrates the usefulness of the SSH approach for the investigation of differential gene expression.

The concerted induction of VCAM-1, MCP-1 and IL-8 expression by TNF has been described in several previous studies [28–31] and is crucially involved in leukocyte activation, adhesion and transendothelial migration. All these genes contain DNA-regulatory elements in their promoter regions that are recognized by transcription factors of the NF-κB/Rel family [8,9]. In resting cells, NF-κB proteins are in an inactive cytosolic form complexed to inhibitor proteins referred to as IκBs. Several IκB proteins have been identified including IκBα, IκBβ, p105, IκBγ, bcl-3 and IκBε [14–16,32]. IκBα, the major inhibitory protein, binds to the p65 subunit of NF-κB through its ankyrin repeats, thereby masking its nuclear localization signal and preventing nuclear import. Upon cell activation, IκBα is rapidly phosphorylated and degraded, which allows the active NF-κB dimer to translocate to the nucleus and to participate in the transactivation of target genes [4–7]. In the present study, we found that IκBα was differentially expressed in HUAEC in response to TNF stimulation. Indeed, the expression of IκBα has been found to be rapidly upregulated by NF-κB itself, providing a negative autoregulatory loop which mediates the replenishment of cytoplasmic IκBα and termination of gene transcription [11–15].

An interesting finding was the identification of syntenin as a TNF-inducible gene in endothelial cells. So far, the transcriptional regulation of syntenin expression has not been studied. Syntenin has been originally identified as an adapter protein binding to the cytoplasmic domain of syndecans, which play a major role in cell-cell and cell-matrix interactions [26]. The four known syndecans have a similar structural organization consisting of a single ectodomain, a membrane spanning region and a cytoplasmic domain [33]. Whereas the ectodomains have little in common, the membrane and small cytoplasmatic regions show extensive structural similarity. The interaction of syndecans with syntenin is mediated by its two PDZ domains. These motifs represent conserved modular structures supporting protein-protein interactions by binding to the C-terminal regions of proteins [34,35]. PDZ domain-containing proteins are able to interact with different proteins such as ion channels, transmembrane receptors, tumor suppressors or GTPase activating proteins [36]. These interactions result in the formation of multimeric protein complexes which mediate receptor clustering or the coupling of proteins to the cytoskeleton and downstream signal components [37].

In our experiments, we found that syntenin expression was upregulated in TNF-stimulated HUAEC, suggesting that it might be regulated in a similar manner as IL-8 and IκBα which are tightly controlled by the NF-κB system. From the early onset of syntenin expression it may be speculated that syntenin in conjunction with syndecan participate in cell-cell interactions, stabilization of transmembrane receptors or exert another signaling function. Likewise, since syntenin has been recently localized also in the apical endocytic compartment [38,39], syntenin may function in regulating the correct localization of membrane receptors or the polarized release of inflammatory mediators. Therefore, further studies have to investigate whether induction of syntenin expression modulates leukocyte adhesion or other proinflammatory functions of the vascular endothelium.

In summary, we show that the technique of SSH allows the rapid identification of differentially expressed genes in the context of gene expression patterns. It should be noted, however, that not all genes that are known to be activated by TNF in endothelial cells, such as for instance IL-1β, were identified by our approach. The expression of the genes detected in the present study was upregulated 4–7-fold com-

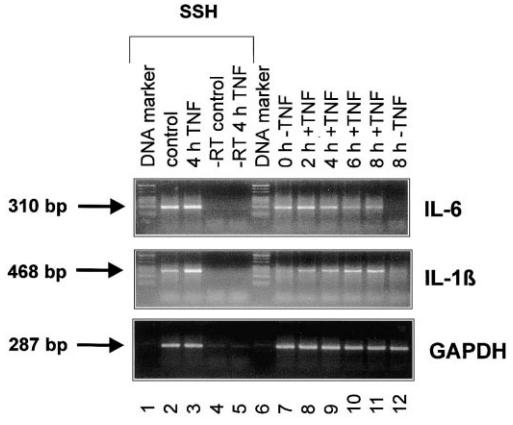

Fig. 5. Expression of IL-6 and IL-1β specific mRNA in response to TNF stimulation. Lanes 2–5 show the PCR amplification products of the RNA which was originally used for SSH, with or without RT reaction. Lanes 7–12 show the expression profiles of an independent time course experiment. HUAEC were incubated in medium alone or treated with 50 ng/ml TNF for the indicated times. Total RNA was isolated and RT-PCR was performed as described in Section 2. The molecular sizes of the resulting PCR products are shown on the left. As a control for the same amount of RNA a RT-PCR with GAPDH primers was performed (lower panel).

pared to unstimulated cells. This may be due to the fact that for the subtraction procedure a five-fold higher amount of the driver cDNA had been added to the tester cDNA. Thus, genes which are only weakly induced by TNF may escape detection. Nevertheless, the SSH technique permits the efficient and rapid cloning of differentially expressed genes and minimizes the problem of isolating false-positive clones.

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