BMC Molecular Biology

Research article

Different effects of antisense RelA p65 and NF-κB1 p50 oligonucleotides on the nuclear factor-κB mediated expression of ICAM-1 in human coronary endothelial and smooth muscle cells

Rainer Voisard*[1], Nicola Huber[1], Regine Baur[1], Milorat Susa[2], Oliver Ickrath[3], Anton Both[3], Wolfgang Koenig[1] and Vinzenz Hombach[1]

Address: [1]Department of Internal Medicine II – Cardiology, University of Ulm, Robert-Kochstrasse 8, D-89081 Ulm, Germany, [2]Institute of Medical Microbiology, University of Ulm, Robert-Kochstrasse 8, D-89081 Ulm, Germany and [3]Katharinen Hospital Stuttgart, Kriegsbergstrasse 60, 70174 Stuttgart, Germany

E-mail: Rainer Voisard* - rainer.voisard@medizin.uni-ulm.de; Nicola Huber - nicola.huber@medizin.uni-ulm.de; Regine Baur - regine.baur@medizin.uni-ulm.de; Milorat Susa - milorat.susa@medizin.uni-ulm.de; Oliver Ickrath - oliver.ickrath@katharinenhospital.de; Anton Both - anton.both@katharinenhospital.de; Wolfgang Koenig - wolfgang.koenig@medizin.uni-ulm.de; Vinzenz Hombach - vinzenz.hombach.@medizin.uni-ulm.de

*Corresponding author

Published: 8 August 2001

BMC Molecular Biology 2001, **2**:7

Received: 10 May 2001
Accepted: 8 August 2001

This article is available from: http://www.biomedcentral.com/1471-2199/2/7

Abstract

Background: Activation of nuclear factor-κB (NF-κB) is one of the key events in early atherosclerosis and restenosis. We hypothesized that tumor necrosis factor-α (TNF-α) induced and NF-κB mediated expression of intercellular adhesion molecule-1 (ICAM-1) can be inhibited by antisense RelA p65 and NF-κB1 p50 oligonucleotides (RelA p65 and NF-κB1 p50).

Results: Smooth muscle cells (SMC) from human coronary plaque material (HCPSMC, plaque material of 52 patients), SMC from the human coronary media (HCMSMC), human endothelial cells (EC) from umbilical veins (HUVEC), and human coronary EC (HCAEC) were successfully isolated (HCPSMC, HUVEC), identified and cultured (HCPSMC, HCMSMC, HUVEC, HCAEC). 12 hrs prior to TNF-α stimulus (20 ng/mL, 6 hrs) RelA p65 and NF-κB1 p50 (1, 2, 4, 10, 20, and 30 μM) and controls were added for a period of 18 hrs. In HUVEC and HCAEC there was a dose dependent inhibition of ICAM-1 expression after adding of both RelA p65 and NF-κB1 p50. No inhibitory effect was seen after incubation of HCMSMC with RelA p65 and NF-κB1 p50. A moderate inhibition of ICAM-1 expression was found after simultaneous addition of RelA p65 and NF-κB1 p50 to HCPSMC, no inhibitory effect was detected after individual addition of RelA p65 and NF-κB1 p50.

Conclusions: The data point out that differences exist in the NF-κB mediated expression of ICAM-1 between EC and SMC. Experimental antisense strategies directed against RelA p65 and NF-κB1 p50 in early atherosclerosis and restenosis are promising in HCAEC but will be confronted with redundant pathways in HCMSMC and HCPSMC.

Introduction

Atherosclerosis is currently considered to be an exaggerated response of the vessel wall to injury characterized by inflammation and fibrocellular proliferation [1]. This

view is supported by the demonstration of abundant macrophages and T lymphocytes in atherosclerotic plaques that accumulate because of adhesion molecule expression [2–4]. Nuclear factor-κB (NF-κB) regulates a variety of genes coding for cytokines [5–9] and adhesion receptors [8], that mediate endothelium-leukocyte adhesion [10]. NF-κB-regulated gene products such as interleukin-lβ (IL-1β), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), and intercellular adhesion molecule-1 (ICAM-1) have been found in tissue specimens of atherosclerotic lesions [1]. Activated NF-κB was identified in smooth muscle cells (SMC), macrophages, and endothelial cells (EC) of human atherosclerotic tissue specimens [11], suggesting a pathophysiologial role of NF-κB in inflammatory and proliferative processes in atherosclerosis [12]. Recently, increased levels of NF-κB were found in a clinical study in humans with unstable angina pectoris [13].

The NF-κB system might be a potential pharmacological target to interfere with chemotactic and adhesive mechanisms within the vessel wall. The prototypic NF-κB dimer, consisting of the subunits RelA p65 and NF-κB1 p50, is present in the cytosol in an inactive state, bound to the inhibitory protein that are collectively termed IκB. Activation of NF-κB by a multitude of stimuli, including inflammatory or lymphoproliferative cytokines, reactive oxygen intermediates and micro-organisms, requires the phosphorylation and proteolytic removal of IκB from the dimeric complex. This is followed by an intermediate translocation of activated NF-κB to the nucleus, where the dimer interacts with regulatory kb elements in promoters and enhancers, thereby controlling inducible gene transcription [8,14]. Recently first reports on inhibitory strategies of NF-κB mediated activities in human EC and SMC have been published [15–19].

ICAM-1 is one of various adhesion molecules that is activated via NF-κB pathway. The expression of ICAM-1 by SMC in human atheroma [20] and in hyperplastic lesions produced by experimental balloon injury [21] indicates that augmented ICAM-1 expression constitutes a marker of SMC activation of considerable in vivo relevance. It has been demonstrated that TNF-α increases the expression of ICAM-1 in human arterial SMC from peripheral [22] and coronary arteries [23,24] in a time and dose dependent manner. Recently our laboratory has reported [25] that high dose aspirin (5 mM) inhibits expression of ICAM-1 in human coronary vascular cells. In order to investigate a more specific inhibition of ICAM-1 expression we analysed the effect of antisense RelA p65 and NF-κB1 p50 oligonucleotides on the NF-κB-mediated expression of ICAM-1.

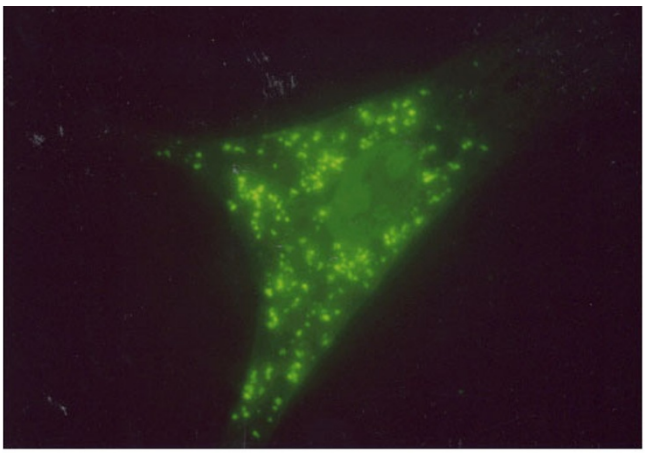

Figure 1
Intracellular uptake of fluorosense oligonucleotides in HCMSMC. Fluorescence microscopy, magnification × 1625.

Results

Intracellular uptake of FITC-labeled antisense

The intracellular uptake of fluorosense oligonucleotides in HUVEC, HCAEC, HCMSMC (Fig. 1), and HCPSMC was confirmed with fluorescence microscopy and flow cytometry. After fluorescence microscopical examination of HUVEC, HCAEC, HCMSMC, and HCPSMC the uptake of fluoresense started 1 hr after incubation. The maximal uptake was reached after 8 hrs, this level was kept for 24 and 48 hrs after incubation.

In flow cytometry examination baseline fluorescence was 2.77 in HUVEC, 4.18 in HCAEC, 1.88 in HCMSMC, and 4.68 in HCPSMC. 18 hrs after adding of fluorosense oligonucleotides the fluorescence intensity was increased to 82.03 and 89.11 in HUVEC and HCAEC (Fig. 2A,B), respectively to 129.78 and 67.58 in HCMSMC and HCPSMC (Fig. 2C,D).

Impact of antisense on TNF-α mediated expression of ICAM-1 in HUVEC

Addition of antisense NF-κB1 p50 oligonucleotide to HUVEC caused only a very small inhibition of the TNF-α induced expression of ICAM-1 (Fig. 4A). In HUVEC the expression of ICAM-1 was 100, 93, and 91% after incubation with antisense NF-κB1 p50 oligonucleotide in a concentration of 1, 2, and 4 μM/mL and 86, 91, and 93% after incubation with antisense NF-κB1 p50 oligonucleotide in a concentration of 10, 20, 30 μM/mL. Incubation of HUVEC with antisense RelA p65 oligonucleotide caused a moderate reduction of the expression of ICAM-1 (Fig. 4A). Expression of ICAM-1 was 66, 71, and 75% after incubation with antisense RelA p65 oligonucleotide in a concentration of 1, 2, and 4 μM/mL and 75, 89, 77% after incubation with antisense RelA p65 oligonucleotide

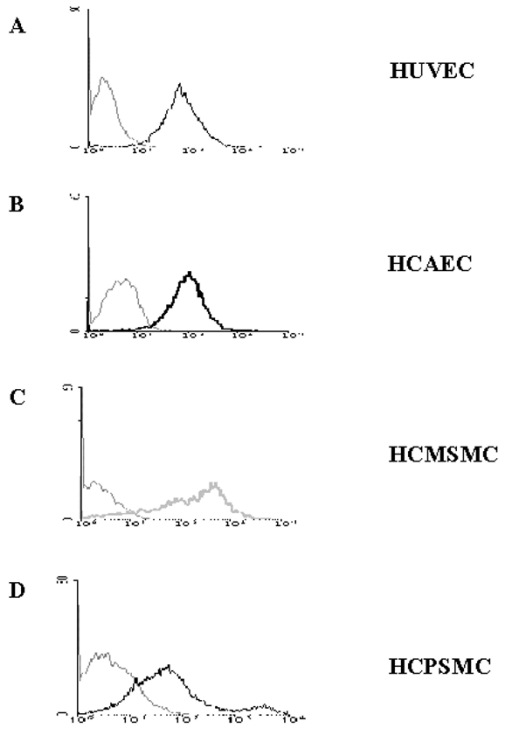

Figure 2
Cytoflow-detection of intracellular FITC-labeled oligonucleotides 18 hrs after incubation: (A) HUVEC, (B) HCAEC, (C) HCMSMC, (D) HCPSMC. Histogramms: x-axis = relative fluorescence, y-axis = cell number.

in a concentration of 10, 20, and 30 μM/mL. Simultaneous adding of antisense RelA p65 and NF-κB1 p50 oligonucleotides to HUVEC caused a dose dependent inhibition of the expression of ICAM-1. Expression of ICAM-1 was 90, 80, 63, and 49% after incubation with antisense RelA p65 and NF-κB1 p50 oligonucleotides in a concentration of 1, 2, 4 and 10 μM/mL. The further increase of the concentration to 20 and 30 μM/mL did not further increase the inhibitory effect. The expression of ICAM-1 was 61% after incubation with 20 μM/mL and 53% after incubation with 30 μM/mL (Fig. 4A, Fig. 3A,B). Mismatched antisense RelA p65 and NF-κB1 p50 oligonucleotides did not affect the expression of ICAM-1 in HUVEC, no matter whether added individually or simultaneously.

Impact of antisense on TNF-α mediated expression of ICAM-1 in HCAEC

Addition of the oligonucleotide antisense NF-κB1 p50 oligonucleotides to cultures of HCAEC had no effect on the expression of ICAM-1 in HCAEC (Fig. 4B). The expression of ICAM-1 was 102, 100, and 100% after incubation with antisense NF-κB1 p50 oligonucleotides in a concentration of 1, 2, and 4 μM/mL and 95, 105, and 105% after incubation with antisense NF-κB1 p50 oligonucleotides in a concentration of 10, 20, and 30 μM/mL. After addition of antisense RelA p65 oligonucleotides to cultures of HCAEC the expression of ICAM-1 was moderately reduced. The expression of ICAM-1 was 65, 74, and 74% after incubation with antisense RelA p65 oligonucleotides in a concentration of 1, 2, and 4 × μM/mL and 74, 79, and 82% after incubation with antisense RelA p65 oligonucleotides in a concentration of 10, 20, and 30 μM/mL (Fig. 4B). Simultaneous incubation of HCAEC with antisense RelA p65 and NF-κB1 p50 oligonucleotides inhibited the expression of ICAM-1 in a strictly dose dependent manner. The expression of ICAM-1 was 102, 83, and 85% after incubation with antisense RelA p65 and NF-κB1 p50 oligonucleotides in a concentration of 1, 2, and 4 μM/mL and 70, 42, and 38% after incubation with antisense RelA p65 and NF-κB1 p50 oligonucleotides in a concentration of 10, 20, and 30 μM/mL (Fig. 4B, 3C,D). Mismatched antisense RelA p65 and NF-k-B1 p50 oligonucleotides did not affect the expression of ICAM-1 in HCAEC, no matter whether added individually or simultaneously.

Impact of antisense on TNF-α mediated expression of ICAM-1 in HCMSMC

In HCMSMC adding of antisense NF-κB1 p50 oligonucleotides caused a moderate increase of the expression of TNF-α induced expression of ICAM-1 (Fig. 4C). The expression of ICAM-1 was 117, 122, and 130% after incubation with antisense NF-κB1 p50 oligonucleotides in a concentration of 1, 2, and 4 μM/mL and 117, 113, and 135% after incubation with antisense NF-κB1 p50 oligonucleotides in a concentration of 10, 20, 30 μM/mL. Antisense RelA p65 oligonucleotides had a small inhibitory effect on the expression of ICAM-1 in HCMSMC. The only exception was the maximal concentration of antisense RelA p65 oligonucleotides (30 μM/mL) with a neutral effect. The expression of ICAM-1 was 81, 91, and 86% after incubation with antisense RelA p65 oligonucleotides in a concentration of 1, 2, and 4 μM/mL. After addition of antisense RelA p65 oligonucleotides in a concentration of 10 and 20 μM/mL the expression of ICAM-1 was 86 and 81% (Fig. 4C). After adding of antisense RelA p65 oligonucleotides in the maximal concentration of 30 μM/mL no inhibitory effect on the surface expression of ICAM-1 was detected. Simultaneous incubation of HCMSMC with antisense RelA p65 and NF-κB1 p50 oligonucleotides caused a small stimulatory effect on the expression of ICAM-1 in HCMSMC. The percentage of HCMSMC with positive expression of ICAM-1 was 103, 100, and 110% after incubation with antisense RelA p65

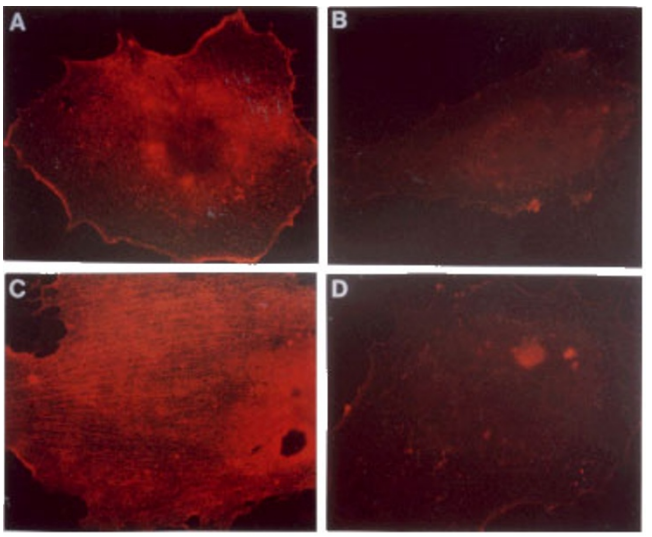

Figure 3
Effect of antisense Rel A p65 and NF-κB1 p50 oligonucleotides on expression of ICAM-1 in HUVEC (B) and HCAEC (D) in comparison to controls with mere TNF-α stimulus (HUVEC: A, HCAEC: C).

and NF-κB1 p50 oligonucleotides in a concentration of 1, 2, and 4 μM/mL and 110, 103, and 107% after incubation with antisense RelA p65 and NF-κB1 p50 oligonucleotides in a concentration of 10, 20, and 30 μM/mL (Fig. 4C). Mismatched antisense RelA p65 and NF-κB1 p50 oligonucleotides did not affect the expression of ICAM-1 in HCMSMC, no matter whether added individually or simultaneously.

Impact of antisense on TNF-α mediated expression of ICAM-1 in HCPSMC

Addition of antisense NF-κB1 p50 oligonucleotides of HCPSMC caused a small reduction of the expression of ICAM-1 (Fig. 4D). The expression of ICAM-1 was 100, 92, and 90% after incubation with antisense NF-κB1 p50 oligonucleotides in a concentration of 1, 2, and 4 μM/mL and 90, 95, and 92% after incubation with antisense NF-κB1 p50 oligonucleotides in a concentration of 10, 20, and 30 μM/mL. Incubation with antisense RelA p65 oligonucleotides had a moderate stimulatory effect on the expression of ICAM-1 (Fig. 4D). The expression of ICAM-1 was 132, 129, and 113% after incubation with antisense p65 oligonucleotides in a concentration of 1, 2, and 4 μM/mL and 116, 129, and 107% after incubation with antisense RelA p65 oligonucleotides in a concentration of 10, 20, and 30 μM/mL. Simultaneous adding of antisense RelA p65 and NF-κB1 p50 oligonucleotides to HCPSMC had a dose dependent inhibitory effect on the expression of ICAM-1. The expression of ICAM-1 was 88, 85, and 91% after incubation with antisense RelA p65 and NF-κB1 p50 oligonucleotides in a concentration of 1, 2, and 4 μM/mL and 73, 73, and 70% after incubation with antisense RelA p65 and NF-κB1 p50 oligonucleotides in a concentration of 10, 20, and 30 μM/mL (Fig. 4D). Mismatched antisense RelA p65 and NF-κB1 p50 oligonucleotides did not affect the expression of ICAM-1 in HCPSMC, no matter whether added individually or simultaneously.

Discussion

In this study we investigated the role of RelA p65 and NF-κB1 p50 for the TNF-α mediated expression of ICAM-1 in HUVEC and human coronary vascular cells. The major findings of the study are: 1) intracellular uptake of antisense RelA p65 and NF-κB1 p50 oligonucleotides was confirmed by the detection of FITC-labeled antisense RelA p65 and NF-κB1 p50 oligonucleotides in the intracellular space. 2) In HUVEC and HCAEC simultaneous incubation with antisense RelA p65 and NF-κB1 p50 oligonucleotides caused a dose dependent inhibition of ICAM-1 expression. The inhibitory effect was almost doubled if compared to the effect after selective addition of antisense RelA p65 oligonucleotides, selective incubation with antisense NF-κB1 p50 oligonucleotides had no effect. 3) Simultaneous incubation with antisense RelA p65 and NF-κB1 p50 oligonucleotides exhibited a moderate inhibitory effect on expression of ICAM-1 in HCPSMC but no effect in HCMSMC. No inhibitory effect was seen after selective addition of antisense RelA p65 and NF-κB1 p50 oligonucleotides to HCMSMC and HCPSMC.

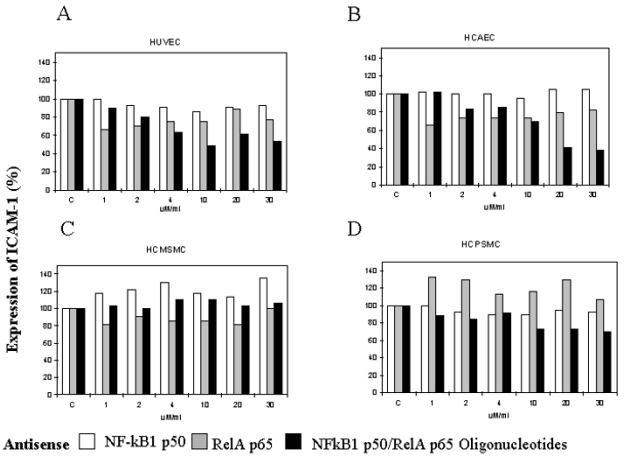

Figure 4
(A) Effect of antisense Rel A p65 and NF-κB1 p50 oligonucleotides on expression of ICAM-1 α in HUVEC, (B) HCAEC, (C) HCMSMC, (D) HCPSMC. Graphic presentation of the inhibitory effect in percent in relation to the stimulatory effect of TNF-α.

Effects of antisense p65 and p50 in HUVEC and HCAEC

Recently it has been reported that a decoy double-stranded oligonucleotides containing a high affinity kb sequence can specifically inhibit NF-κB-dependent transcription of gene products [29,30]. Lockyer et al. [15] and Gawaz et al. [16] demonstrated that in HUVEC the expression of ICAM-1 can be inhibited via the adding of the inhibitory protein IκB. The effect of antisense RelA p65 and NF-κB1 p50 oligonucleotides to the expression of adhesion molecules in cultures of HUVEC and HCAEC has not yet been described. After adding simultaneously antisense RelA p65 and NF-κB1 p50 oligonucleotides the expression of ICAM-1 in HUVEC and HCAEC was inhibited in a dose dependent manner. The inhibitory effect was almost 50% in HUVEC and even more than 40% in HCAEC. The stronger effect in HCAEC can be partially explained by the fact that the stimulatory effect of TNF-α is stronger in HCAEC in comparison to HUVEC [23]. Selective addition of mere antisense RelA p65 had a moderate inhibitory effect, no effect was documented after selective adding of antisense NF-κB1 p50. A predominance of RelA p65 in the dimers of the NF-κB complex has already been suggested by Brand et al. [11]. The inhibition of ICAM-1 expression in HUVEC and HCAEC after simultaneous addition of antisense RelA p65 and NF-κB1 p50 was almost twice as high as the effect after addition of mere antisense RelA p65. Therefore it can be speculated that a simultaneous administration of antisense RelA p65 and NF-κB1 p50 is necessary if a sufficient inhibition of NF-κB mediated activities shall be achieved.

Effects of antisense p65 and p50 in HCMSMC and HCPSMC

Although many authors describe the activation of NF-κB in endothelial cells, relatively few data exist concerning the activation of NF-κB in SMC. Lawrence et al. [31] demonstrated that bovine SMC exhibit basal, constitutive NF-κB activity in vitro, Cercek et al. [32] found an increased activity of NF-κB after balloon injury in rat iliofemoral arteries. First investigations of NF-κB activation in human arterial SMC were reported by Bourcier et al. [33], Marayama [34], and Hishikawa et al. [17]. In an earlier report we have demonstrated that the expression of ICAM-1 in HCMSMC and HCPSMC is increased in a time and dose dependent manner after treatment with TNF-α in a concentration of 2.5 – 20 ng/mL [23].

Recently Katsuyama et al. [35] reported that in rat vascular smooth muscle cells a strong inhibition of NF-κB mediated effects can be achieved by dexamethasone or aspirin. Only few groups investigated strategies to inhibit activation of NF-κB in human vascular SMC. Staels et al. [18] reported that the activation of NF-κB in human aortic SMC can be inhibited by peroxisome proliferator-activated alpha receptors (PPARalpha), Speir et al. [19] demonstrated in HCMSMC that aspirin inhibits the cytomegalo-virus-induced activation of NF-κB.

The group of Hishikawa et al. [17] recently demonstrated with electrophoretic mobility shift assays that in HCMSMC the TNF-α induced activation of NF-κB can be inhibited by antisense RelA p65 and NF-κB1 p50 oligonucleotides. If the NF-κB system is sufficiently inhibited it should be expected that NF-κB mediated effects are inhibited as well. In the study presented we added antisense RelA p65 and NF-κB1 p50 oligonucleotides to HCMSMC and HCPSMC and investigated the expression of ICAM-1. However TNF-α induced and NF-κB mediated expression of ICAM-1 was not inhibited in HCMSMC, only a moderate inhibitory effect was seen in HCPSMC. Different inhibitory effects in HCMSMC and HCPSMC however are difficult to explain because the stimulatory effect of TNF-a on the expression of ICAM-1 is almost equal in HCPSMC and HCMSMC [23]. Furthermore in HCPSMC no inhibitory effect was found after selective addition of antisense RelA p65 or NF-κB1 p50 oligonucleotides. Therefore it is likely that the moderate inhibitory effect found after addition of antisense RelA p65 and NF-κB1 p50 oligonucleotides to HCPSMC is a nonspecific effect.

Keeping in mind the negative effects in HCMSMC and the non specific effects in HCPSMC it is allowed to speculate that in HCPSMC and HCMSMC activation of ICAM-1 is triggered via different pathways, probably via different members of the NF-κB family [36]. This hypothesis is supported by Lindner et al. [37] investigating the role of the NF-κB system after balloon catheter injury in the rat carotid artery. Lindner and colleagues [37] reported that in rat vascular SMC not only high levels of NF-κB1 p50 and RelA p65 but although high levels of NF-κB2 p52, c-Rel, and RelB were expressed.

Conclusions

The results point out that in HUVEC and HCAEC simultaneous antisense therapy with NF-κB1 p50 and RelA p65 oligonucleotids strongly inhibits activation NF-κB mediated expression of ICAM-1. After individual addition of antisense NF-κB1 p50 and RelA p65 oligonucleotides to HUVEC and HCAEC only RelA p65 had an inhibitory effect on TNF-α mediated expression of ICAM-1. However the superiority of the combined therapy was demonstrated by the fact that the inhibitory potential was almost doubled after the simultaneous therapy with RelA p65 and NF-κB1 p50.

In HCMSMC and HCPSMC the negative or nonspecific effects of antisense RelA p65 and NF-κB1 p50 oligonucleotides on TNF-α mediated expression of ICAM-1 indi-

cate the existence of redundant pathways. In further studies it should be elucidated whether HCMSMC and HCPSMC express other members of the NF-κB family as well, such as NF-κB2 p52, c-Rel, and RelB. If these molecules are expressed in HCMSMC and HCPSMC, all of these single pathways have to be blocked for a sufficient inhibition of NF-κB-related effects.

Materials and Methods

Cell isolation, identification, and cultivation

Endothelial cells (EC) were isolated from fresh human umbilical cord veins (HUVEC) obtained after vaginal delivery by enzymatic disaggregation with collagenase/dispase as previously described [26]. EC from human coronary arteries (HCAEC) were purchased from Clonetics-Bio Whittaker. HUVEC and HCAEC were identified as EC by their by nonoverlapping cobblestone morphology and by positive immunofluorescence with anti-von Willebrand factor antibodies (Dakopatts, Hamburg, Germany). EC were cultured in Endothelial Basal Medium (EBM, Clonetics-Bio Whittaker), supplemented with bovine brain extract (12 μg/mL), epidermal growth factor (10 ng/mL), fcs (5%), gentamycin (50 μg/mL), amphotericin (50 ng/mL), and hydrocortison (1 μg/mL). EC were subcultured by trypsinization [26] and cells up to passage 6 were used for the investigations. SMC from the human coronary media (HCMSMC) were purchased from Clonetics-Bio Whittaker (Venders, Belgium). Atherosclerotic plaque material was extracted from coronary arteries of 52 patients with a Simpson atherectomy device. Immediately after extraction the specimens were transferred to sterile glass flasks containing N-(2-hydroxyethyl)piper-N-(2-ethane sulfonic acid)-buffered Dulbecco's modified Eagle medium (15 mM; Gibco, Eggenhausen, Germany) and transported within 3 h to the cell culture laboratory. SMC from human coronary plaque material (HCPSMC) were isolated with a collagenase/elastase enzyme mixture (Roche Diagnostics, Mannheim, Germany) [27]. HCPSMC and HCMSMC were confirmed as SMC by their typical "hill and valley" growth pattern and their positive staining with antibodies specific for smooth muscle α-actin (Progen, Heidelberg, Germany), as described [27]. HCPSMC and HCMSMC were cultured in passage 2 and 3 in Smooth Muscle Cell Basal Medium (SmBM, Clonetics-Bio Whittaker), supplemented with 5% fetal calf serum (fcs), insulin (5 μg/mL), fibroblast growth factor (2 ng/mL), epidermal growth factor (0,5 ng/mL), gentamycin (50 mg/mL), and amphotericin (50 ng/mL).

All cell incubations were done at 37°C in a humidified atmosphere of air containing 5% C02. Tissues were collected with the patients' informed consent and approval by the Ethical Committee of the University of Ulm.

Oligonucleotides

Intracellular uptake of oligonucleotides was tested with fluorescence microscopy and flow cytometry investigations. For fluorescence microscopy HUVEC, HCAEC, HCMSMC, and HCPSMC were seeded on 16-well dishes on glass slides (Nalge-Nunc, Hamburg, Germany) in a density of 2–3 × 103 Zellen × cm-2. Cells were incubated with fluorescein-labeled antisense oligonucleotides (fluorosense; form: phosphothioate DNA-Na-salt diluted with oligo-buffer) for a period of 1, 2, 4, 8, 24, and 48 hrs with a stock solution (5 nmol/5 μl; Biognostik, Göttingen, Germany). For the investigations an immunofluorescence microscope was used.

For flow cytometry investigations 2 × 105 HUVEC, HCAEC, HCMSMC, and HCPSMC were seeded in petri dishes (28 cm2) and the stock solution was added for a period of 18 hrs to the cultures. After 18 hrs cells were washed twice with PBS, detached by trypsin treatment, and suspended in phosphate-buffered saline, pH 7.2, containing 1% of bovine serum albumin (BSA) with 0.2% of sodium azide at 4°C. This buffer was used in all subsequent steps. Cells (100% gated) were analyzed immediately with a fluorescence-activated cell sorter (FACScan, Becton Dickinson, Heidelberg, Germany, Macintosh System Software 7.1.0).

Antisense RelA p65 and NF-κB1 p50 oligonucleotides is the revise complement of a target sequence as described by Ruben et al. [28]. The form is a phosphothioate DNA-Na-salt, lyophilized and diluted with oligo-buffer (Biognostik). Antisense RelA p65 and NF-κB1 p50 oligonucleotides are added for a period of 18 hrs in a concentration of, 1, 2, 4, 10, 20, and 30 μM. As controls mismatched antisense RelA p65 and NF-κB1 p50 oligonucleotides were used. Form and concentration is identical as described for antisense RelA p65 and NF-κB1 p50 oligonucleotides.

TNF-α induced expression of ICAM-1

Surface expression of ICAM-1 was investigated with indirect immunofluorescence microscopy. HUVEC, HCAEC, HCMSMC, and HCPSMC were seeded on round coverslips into 6-well plates (Falcon/Becton-Dickinson, Heidelberg, Germany). 18 hrs before fixation in 4% paraformaldehyde antisense RelA p65 and NF-κB1 p50 oligonucleotides and mismatched controls were added, 6 hrs before fixation cultures were stimulated with recombinant human TNF-α (20 ng/mL; Sigma, Deisenhofen, Germany). Human ICAM-1 was detected using monoclonal anti-ICAM-1 antibodies (clone 84H10, 2 μg/mL, Dianova Immunotech, Hamburg, Germany). Tetrarhodamine-isothiocyanate (TRITC)-labeled secondary antibodies were purchased from Dianova Immunotech. In 100 cells the expression of ICAM-1 was analysed in duplicate by two independent observers according the cri-

teria negative, low and strong. Since both low and negative staining were found as basic expression of ICAM-1 in untreated controls, only a strong positive staining was rated as positive expression of ICAM-1. The inhibitory effect of antisense RelA p65 and NF-κB1 p50 oligonucleotides was calculated in relation to the relative stimulatory effect of TNF-α. For the investigations an immunofluorescence microscope was used (100 cell per concentration, magnification × 100).

Acknowledgments

The authors would like to express their gratitude to Roland Schmid, Department of Internal Medicine II, University of Ulm, Germany, for excellent support in the design of the study and for critical discussion of the manuscript and to Rolf Kreienberg, Department of Gynaecology and Obstetrics, University of Ulm, for providing umbilical veins for isolation of HUVEC.

References

1. Ross R: **Atherosclerosis – an inflammatory disease.** *N Engl J Med* 1999, **340**:115-126
2. Jonasson L, Holm J, Skalli 0, Bondjers G, Hansson GK: **Regional accumulation of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque.** *Arteriosclerosis* 1986, **6**:131-138
3. Sato T, Takebayashi S, Kohehi K: **Increased subendothelial infiltration of the coronary arteries with monocytes/macrophages in patients with unstable angina.** *Atherosclerosis* 1995, **68**:191-197
4. Mazzone A, De Servi S, Ricevuti G, Mazzucchelli I, Fossati G, Pasotti D, Bramucci E, Angoli L, Marsico F, Specchia G, Notario A: **Increased expression of neutrophil and monocyte adhesion molecules in unstable coronary artery disease.** *Circulation* 1993, **88**:358-363
5. Collins T: **Endothelial nuclear factor-κB and the initiation of the atherosclerotic lesion.** *Lab Invest* 1993, **68**:499-508
6. Baeuerle PA: **The inducible transcription activator NF-κB: regulation by distinct protein subunits.** *Biochim Biophys Acta* 1991, **1072**:63-80
7. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T: **Transcriptional regulation of endothelial cell adhesion molecules: NF-κB an cytokine-inducible enhancers.** *FASEB J* 1995, **9**:899-909
8. Thanos D, Maniatis T: **NF-κB: a lesson in family values.** *Cell* 1995, **80**:529-532
9. Kupatt C, Habazettl H, Goedecke A, Wolf DA, Zahler S, Boekstegers P, Kelly RA, Becker BF: **Tumor necrosis factor-α contributes to ischemia- and reperfusion-induced endothelial activation in isolated hearts.** *Circ Res* 1999, **84**:392-400
10. Defillipi P, Silengo L, Tarone G: **Regulation of adhesion receptor expression in endothelial cells.** *Curr Top Microbiol Immunol* 1993, **184**:87-98
11. Brand K, Page S, Rogler G, Bartsch A, Brandl R, Knuechel R, Page M, Kaltschmidt C, Baeuerle PA, Neumeier D: **Activated transcription factor nuclear factor-κB is present in the atherosclerotic lesion.** *J Clin Invest* 1996, **97**:1715-1722
12. Brand K, Page S, Walli AK, Neumeier D, Baeuerle PA: **Role of NF-κB in atherogenesis.** *Exp Physiol* 1997, **82**:297-304
13. Ritchie ME: **Nuclear factor-κB is selctively and markedly activated in humans with unstable angina pectoris.** *Circulation* 1998, **98**:1707-1713
14. Baldwin ASJr: **The NF-κB and I-κB proteins: New discoveries and insights.** *Annual Review of Immunology* 1996, **14**:649-681
15. Lockyer JM, Colladay JS, Alperin-Lea WL, Hammond T, Buda AJ: **Inhhibition of nuclear factor-κB mediated adhesion molecule expression in human endothelial cells.** *Circ Res* 1998, **82**:314-320
16. Gawaz M, Neumann FJ, Dickfeld T, Koch W, Laugwitz KL, Adelsberger H, Langenbrink K, Page S, Neumeier D, Schomig A, Brand K: **Activated platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelilal cells.** *Circulation* 1998, **98**:1164-1171
17. Hishikawa K, Oemar BS, Yang Z, Luscher TF: **Pulsatile stretch stimulates superoxide production and activates nuclear factor-κB in human coronary smooth muscle.** *Circ Res* 1997, **81**:797-803
18. Staels B, Koenig W, Habib A, Merval R, Lebret M, Torra IP, Delerive P, Fadel A, Chinetti G, Fruchart JC, Najib J, Maclouf J, Tedgui A: **Activation of human aortic smooth-muscle cells is inhibited by PPARalpha but not by PPARgamma activators.** *Nature* 1998, **393**:790-793
19. Speir E, Yu ZX, Ferrans VJ, Huang ES, Epstein SE: **Aspirin attenuates cytomegalovirus infectivity and gene expression mediated by cyclooxygenase-2 in coronary artery smooth muscle cells.** *Circ Res* 1998, **83**:210-216
20. Poston RN, Haskard DO, Coucher JR, Gall NP, Johnson-Tidey RR: **Expression of intercellular adhesion molecule-1 in atherosclerotic plaques.** *Am J Pathol* 1992, **140**:665-673
21. Tanaka H, Sukhova GK, Swanson SJ, Clinton SK, Ganz P, Cybulsky MI, Libby P: **Sustained activation of vascular cells and leukocytes in the rabbit aorta after balloon injury.** *Circulation* 1993, **88**:1788-1803
22. Couffinhal T, Duplàa C, Labat L, Lamaziere JMD, Moreau C, Printseva 0, Bonnet J: **Tumor necrosis factor-α stimulates ICAM-1 expression in human vascular smooth muscle cells.** *Arteriosclerosis and Thrombosis* 1993, **13**:407-414
23. Voisard R, Oβwald M, Baur R, Jakob U, Susa M, Mattfeldt T, Hemmer W, Hannekum A, Koenig W, Hombach V: **ICAM-1 expression in human coronary endothelial and smooth muscle cells after stimulation with TNF-α.** *Coron Artery Dis* 1998, **9**:737-745
24. Voisard R, Voglic S, Baur R, Susa M, Koenig W, Hombach V: **Leukocyte attack in a 3D human coronary in vitro model.** *Coron Artery Dis* 2001, **12**:401-411
25. Voisard R, Fischer R, Oβwald M, Voglic S, Baur R, Susa M, Koenig W, Hombach V: **Aspirin (5 mM) inhibits key events of early atherosclerosis and restenosis in a 3D human coronary in vitro model.** *Circulation* 2001, **103**:1688-1694
26. Jaffe EA, Nachman RL, Becker CG, Minick CR: **Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria.** *J Clin Invest* 1973, **52**:2745-2756
27. Dartsch PC, Voisard R, Betz E: **In vitro growth characteristics of human atherosclerotic plaque cells: Comparison of cells from primary stenosing and restenosing lesions of peripheral and coronary arteries.** *Res Exp Med* 1990, **190**:77-87
28. Ruben SM, Dillon PJ, Schreck R, Henkel T, Chen CH, Maher M, Baeuerle PA, Rosen CA: **Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NF-κB.** *Science* 1991, **251**:1490-1493
29. Bielinska A, Shivdasani RA, Zhang L, Nabel GJ: **Regulation of gene expression with double-stranded phosphorothioate oligonucleotides.** *Science* 1990, **250**:997-1000
30. Ech SL, Perkins ND, Carr DP, Nabel GJ: **Inhibition of phorbol ester-induced cellular adhesion by competitive binding of NF-κB in vivo.** *Mol Cell Biol* 1993, **13**:6530-6536
31. Lawrence R, Chang LJ, Siebenlist U, Bressler P, Sonenshein GE: **Vascular smooth muscle cells express a constitutive NF-κB like activity.** *J Biol Chem* 1994, **269**:28913-28918
32. Cercek B, Yamashita M, Dimayuga P, Zhu J, Fishbein MC, Kaul S, Shah PK, Nilsson J, Regnstrom J: **Nuclear factor-κB activity and arterial response to balloon injury.** *Atherosclerosis* 1997, **131**:59-66
33. Bourcier T, Sukhova G, Libby P: **The nuclear factor-κB signaling pathway participates in dysregulation of vascular smooth muscle cells in vitro and in human atherosclerosis.** *J Biol Chem* 1997, **272**:15817-15824
34. Maruyama I, Shigeta K, Miyahara H, Nakajima T, Shin H, Die S, Kitajima I: **Thrombin activates NF-κB through thrombin receptor and results in proliferation of vascular smooth muscle cells: role of thrombin in atherosclerosis and restenosis.** *Ann N Y AcadSci* 1997, **811**:429-436
35. Katsuyama K, Shichiri M, Kato H, Imai T, Mammo F, Hirata Y: **Differential inhibitory actions by glucocorticoid and aspirin on cytokine-induced nitric oxide production in vascular smooth muscle cells.** *Endocrinology* 1999, **140**:2183-2190
36. Siebenlist U, Franzoso G, Brown K: **Structure, regulation and function of NF-κB.** *Annu Rev Cell Biol* 1994, **10**:405-455
37. Lindner V: **The NF-κB and IκB system in injured arteries.** *Pathobiology* 1998, **66**:311-320