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Endothelin-1 Induces Functionally Active CD40 Protein via Nuclear Factor-κB in Human Vascular Smooth Muscle Cells

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Abstract

A plethora of evidence supports a link between inflammation and atherogenesis. The vasoactive peptide endothelin-1 (ET-1) has both proatherogenic and proinflammatory properties. The CD40-CD154 signaling pathway exhibits a direct influence on atherogenesis. We therefore tested the hypothesis that ET-1 induces CD40 in human vascular smooth muscle cells (SMC). ET-1 concentration-dependently stimulated CD40 protein in SMC. The specific ET-A-receptor antagonist BQ-123 prevented CD40 induction demonstrating receptor specificity of the ET-1 effect. Experiments with PI-1, an inhibitor of the IκB-α-degrading proteasome complex, demonstrated involvement of the transcription factor NF-κB in ET-1-induced CD40 expression. Specific decoy oligodeoxynucleotides with the consensus binding sequence for NF-κB and AP-1 supported a NF-κB-dependent and AP-1-independent induction pathway. Functional relevance of ET-1-induced CD40 expression was demonstrated by an increase in IL-6 secretion after stimulation with CD154 of cells preactivated with ET-1. The data show a link between a proatherogenic vasoactive peptide and cell-cell contact mediated inflammatory pathways and may implicate novel therapeutic options for vascular disease.

Key words: endothelin-1; atherosclerosis; smooth muscle cell; transcription factors; CD40

Abbreviations: ET-1 = endothelin-1; NF-κB = nuclear factor-κB; AP-1 = activator protein-1; SMC = smooth muscle cells; PI-1 = proteasome inhibitor-1; TNFα = tumor necrosis factor α; IFN-γ = interferon-γ; LDL = low-density lipoprotein

Introduction

A body of evidence supports a link between inflammation and atherogenesis [1-3]. The vasoactive peptide ET-1 is known as a proatherogenic factor not only because of its vasoconstrictive and mitogenic properties [4-6] but also through its capability to induce an inflammatory response in human vascular SMC [7].

CD40 is a 45-50-kDa membrane glycoprotein which belongs to the tumor necrosis factor (TNF) superfamily. In addition to monocytes and macrophages, human vascular endothelial cells, smooth muscle cells and dendritic cells in atherosclerotic plaques also express CD40 [8, 9]. The ligand for CD40, CD40L or CD154, is a 39-kDa glycoprotein which was originally thought to be restricted to activated T-cells [10]. More recently, activated platelets were described as another major source of CD154 triggering inflammatory reactions of endothelial cells [11, 12]. Elevated levels of soluble CD154 are present in patients with acute coronary syndromes [13, 14] as well as in patients with chronic heart failure [15]. In both conditions high amounts of circulating or local levels of ET-1 can be found [16-19].

Today CD40-CD154 interactions are recognized as important in the pathogenesis of atherosclerosis. Inhibition of CD40 signaling in mouse models of progressive atheroclerosis resulted in a dramatic reduction of development of atherosclerotic lesions at all [20] or yielded a more stable plaque phenotype [21-23].

Since ET-1 induces CD40 on human monocytes [24] and is a potent inducer of cytokine secretion in human vascular smooth muscle cells [7] the present study investigated if ET-1 stimulates CD40 expression in human SMC as a potentially novel link between inflammatory pathways.

Materials and Methods

Cell preparation and culture

SMC were cultured by explant outgrowth as described [7] from unused portions of human saphenous veins harvested for coronary bypass surgery. For experiments, confluent cells from passages 2 to 5 were growth-arrested in serum-free medium [25].

Determination of CD40 expression with Western blot

Briefly, cell extracts were separated on SDS-PAGE minigels and electrophoretically transferred to Hybond-nitrocellulose membrane (Amersham). Incubation with monoclonal antibodies for CD40 (Santa Cruz) was followed by incubation with secondary antibodies coupled to horseradish peroxidase. Antibody binding was visualized using the ECL chemiluminescence system (Amersham).

RNA isolation and RT-PCR

After extraction of total RNA by RNAzol B (Wak-Chemie) complementary DNA was synthesized from 1 µl samples of total RNA (1 µg) using Moloney murine leukemia virus reverse transcriptase (Fermentas). Specific cDNA from the reverse transcriptase re-

action product was amplified using human CD40 specific primers [26] with following sequences: 5'-CAGAGTTCACTGAAACGGAATGCC-3' and 5'-TGCCTGCCTGTTGCACAACC-3' and GAPDH specific primers with Taq DNA polymerase (Fermentas). The amplification products (380 bp for CD40 and 212 bp for GAPDH) were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining.

Decoy oligodeoxynucleotide technique
Double-stranded decoy oligodeoxynucleotides with the consensus binding sequence for NF-κB and AP-1 were obtained from complementary single-stranded oligodeoxynucleotides with phosphorothioate modifications (MWG Biotech) as described [7]. SMC were preincubated with 10 μmol/L double-stranded oligodeoxynucleotides in serum-free medium for 5 h followed by a stimulation period of 48 h with ET-1 without additional decoy application. Passive uptake of the oligodeoxynucleotides into the cells was confirmed by microscopy using fluorescence-marked oligodeoxynucleotides. The single-strand sequence of the NF-κB oligodeoxynucleotide was 5'-AGTTGAGGGGACTTT-CCCAGGC-3' with underlined letters denoting phosphorothioate-modified bases. The AP-1 sequence was 5'-CGCTTGATGACTCAGCCGGAA-'3.

Determination of IL-6 release by ELISA
Human vascular SMC were plated on gelatine-coated 96-well tissue culture dishes (CoStar, Cambridge, MA, USA) and incubated at 37 °C until confluency. They were kept in insulin-transferrin medium for 2 d before the experiment. For the experiments some of the cells were preincubated with ET-1 for 24 h. Then supernatants were removed, replaced by fresh medium and cells were stimulated with recombinant CD154 for another 24 h. Afterwards supernatants were collected and assayed for IL-6 with an ELISA kit (Endogen) according to the manufacturer's instructions. The assay selectively recognizes IL-6 with a limit of detection of <1pg/ml.

Statistics
Statistical significance among multiple groups was determined using a nonparametric analysis of variance (ANOVA) based on the ranks of data by "the mixed procedure" of the SAS system (SAS Institute Inc., Cary, NC, USA) and was set at $^*p < 0.05$.

RESULTS

Endothelin-1 stimulates CD40 protein expression via ET-A-receptors
ET-1, like IFN-γ (100 U/ml) alone or in combination with TNFα (20 ng/mL) as positive control stimuli, concentration-dependently induced CD40 protein expression by SMC over 24 h. The increase in CD40 expression occured over an ET-1 concentration range between 100 pmol/L and 1 μmol/L (Fig. 1A) with a maximal effect at 10 nmol/L. This is a concentration range similar to that required for stimulation of cytokine release by ET-1 [7]. BQ-123, a highly specific ET-A-receptor antagonist, inhibited the effect (Fig. 1B) whereas the ET-B-receptor antagonist BQ-788 (10 mmol/L each) did not. This suggests that ET-1-induced CD40 expression is specific and mediated by the endothelin receptor subtype ET-A.

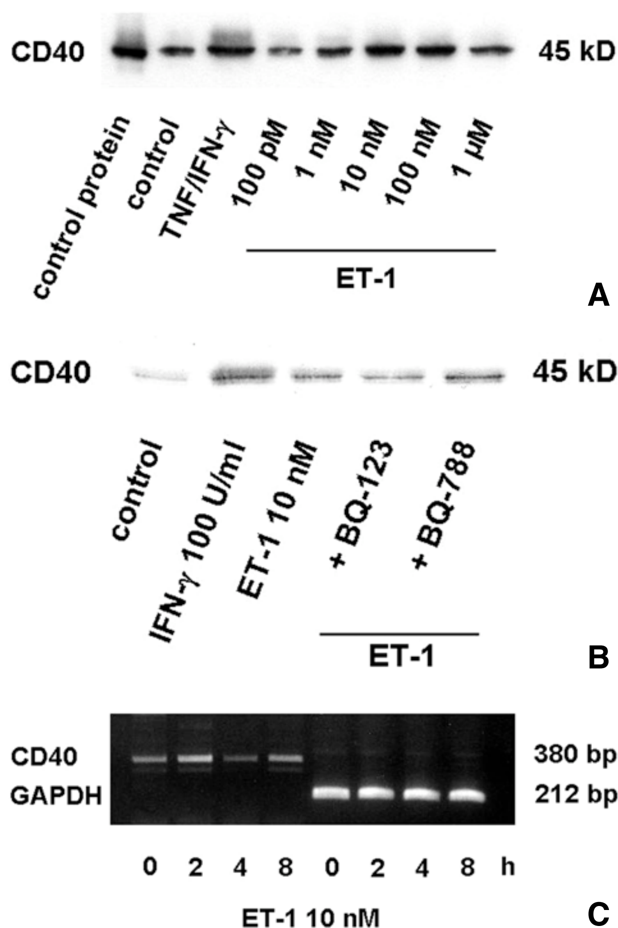

Fig. 1. Endothelin-1 stimulates CD40 expression and CD40-mRNA in human vascular smooth muscle cells. Cells were grown to confluency and growth-arrested in serum-free medium. For the experiment, the medium was replaced by fresh medium with or without ET-1 (100 pmol/L - 1 μmol/L) or the positive control stimuli TNFα (20 ng/mL) and/or INF-γ (100 IU/mL). At the end of the experiment the medium (A, B) was assayed for CD40 content by Western blot (10 mg protein/lane), or mRNA expression was assessed by RT-PCR (C).
(A) Concentration-dependent CD40 release induced by ET-1. CD40 expression peaked after 24 h at maximum at 10 nM ET-1. A 45 kD CD40 control protein on the left hand side of the figure indicates specifity of the band in a representative Western blot. N=3.
(B) Inhibition of ET-1-mediated CD40 release by the specific ET-A-receptor antagonist BQ-123. The specific ET-B-receptor antagonist BQ-788 did not inhibit CD40 expression (10 μmol/L each).
(C) Time-dependent induction of CD40 mRNA by ET-1 assessed by RT-PCR. Results are representative for two independent experiments with cells from different donors.

ET-1 also transiently increased steady-state levels of CD40 mRNA, suggesting regulation of CD40 expression at the pretranslational, presumably transcriptional level (Fig. 1C).

Endothelin-1-induced CD40 expression is NF-κB dependent
Since the promoter of the CD40 gene contains binding sites for the transcription factors NF-κB and AP-1, and ET-1 activates NF-κB and AP-1 in SMC [7, 27] we tested the hypothesis, that ET-1-induced CD40 release depends on NF-κB and/or AP-1. Firstly, the unspecific proteasome inhibitor PI-1 (50 μmol/L) prevented ET-1-induced CD40 expression (Fig. 2A). Secondly, decoy oligodeoxynucleotides (10 μmol/L) with the binding sequences for activated NF-κB prevented the increase in ET-1-induced CD40 expression after 48 hours whereas decoy oligodeoxynucleotides against the transcription factor AP-1 did not (Fig. 2B).

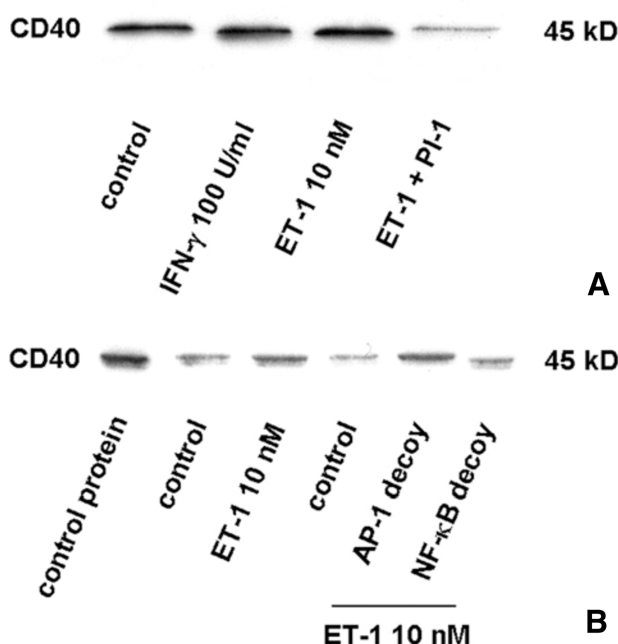

Fig. 2. Endothelin-1 induces CD40 release via NF-κB. Cells were grown to confluency and growth-arrested in serum-free medium. For the experiment, the medium was replaced by fresh medium with or without ET-1 (10 nmol/L).
(A) The effect of ET-1 on CD40 expression was investigated in the presence of PI-1, an inhibitor of NF-κB activation. IFN-γ served as positive control.
(B) Six hours prior to ET-1 stimulation, cells were incubated with an oligodeoxynucleotide containing the binding sequence for activated NF-κB or AP-1 ("decoy", 10 μmol/L each). No additional decoy application during the following 48 h stimulation period with ET-1 (10 nmol/L). Cell extracts were assayed for CD40 protein content by Western blot. N = 3.

Endothelin-1-induced CD40 protein is functionally active
To test the functional relevance of ET-1-increased CD40 expression SMC were prestimulated with ET-1 and subsequently incubated with recombinant CD154 (5 ng/mL). Cells prestimulated with ET-1 significantly secreted more IL-6 than control cells (Fig. 3).

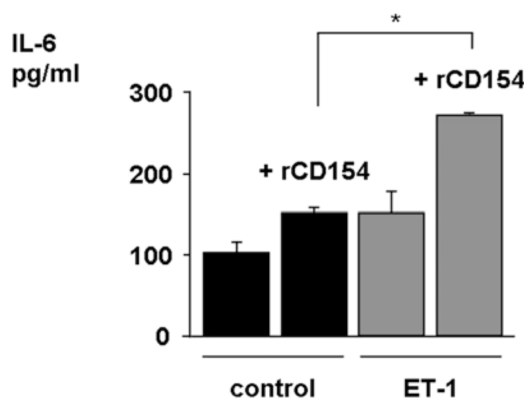

Fig. 3. ET-1-induced CD40 is functionally active. Cells were preincubated for 24 h with out without ET-1 (10 nmol/L). Then the medium was replaced by fresh medium with or without rCD154 (5 ng/mL) for another 24 h. IL-6 concentration in the supernatant was assayed by ELISA. Results are shown as mean + SEM and are representative for 2 independent experiments. $p < 0.05$.

Discussion

The present data demonstrate the ability of ET-1 to increase expression of functionally active CD40 protein on human vascular SMC by a NF-κB-dependent and AP-1-independent pathway in human vascular SMC. The vasoactive peptide ET-1 is known as a proatherogenic risk factor not only because of its vasoconstrictive and mitogenic properties [4-6] but also through its capability to induce an inflammatory response in human vascular SMC. Our *in vitro* data are in keeping with previous reports on CD40 expression in human atherosclerotic plaques [9] and increased levels of ET-1 in culprit lesions of patients with acute coronary syndromes [16, 17]. The described direct ET-A-receptor-mediated effect of ET-1 on CD40 expression may be accompanied by indirect ET-1 effects on locally induced cytokine and chemokine synthesis which also can upregulate CD40 expression in human vascular cells [28, 29]. Upregulation of CD40 in other cellular components of atherosclerotic tissue, for example in human monocytes [24], may lead to a greater binding capacity for its ligand CD154. This can result in secretion of matrix metalloproteinases [30] or tissue factor [9, 31]. Especially activated platelets and CD4+ activated T-cells may serve as a source of soluble CD154 in acute coronary syndromes [12, 13]. T-cells are, through secretion of IFN-γ, potent inducers of CD40 [32]. The importance of CD40-CD154 interactions in atherosclerosis is underlined by experiments using mouse models with progressive atherosclerosis. In atherosclerosis-prone mice lacking CD154 the atherosclerotic lesions contained less lipid and more collagen which resulted in amore stable plaque phenotype [21, 23]. Studies using anti-CD154 antibody treatment in LDL-receptor deficient mice or in ApoE knock-out mice not only demonstrated reduced lesion progression but also changed the composition of atheroma in a fashion that favors plaque stability. To our knowledge no data on human cells exist showing the influence of ET-1 on CD40 expression on human vascular SMC.

A rise in steady-state CD40 mRNA preceeded the increase in CD40 expression. Since the promoter region of CD40 contains NF-κB sites, and our own data demonstrated activation of this proinflammatory tran-

scription factor in SMC by ET-1 [7] we blocked activated NF-κB by specific decoy oligodeoxynucleotides. The NF-κB decoy prevented ET-1-induced CD40 expression. This finding suggests that NF-κB activation is involved in the augmentation of CD40 expression by the vasoactive peptide. Since AP-1 is another transcription factor involved both in ET-1 cell signaling and CD40 expression in SMC [27] comparable experiments were performed with AP-1 decoy oligodeoxynucleotides. Decoys targeted against AP-1 did not inhibit ET-1-induced CD40 protein expression in human SMC demonstrating an activation mechanism independent from AP-1.

Furthermore, ET-1-induced CD40 expression led to an increase in secretion of the proinflammatory cytokine IL-6 after ligation with CD154 compared to SMC not preactivated by ET-1. This finding underlines the functional activity and relevance of the ET-1-induced CD40 protein. Thus, ET-1 may act as an inducer of cell-cell-contact dependent inflammatory pathways via CD40 expression besides its known function as a local regulator of vascular tone. Pharmacologic interference with the CD40-CD154 system may block atherosclerosis also in man. Diabetics show an upregulated CD40-CD154-system [33] and an enhanced surface expression of certain collagen receptors on platelets that mediate CD154 release [34]. Treatment with rosiglitazone an antidiabetic agent of the thiazolidinedione group reduced sCD154 serum levels in type 2 diabetics [35]. Lipid lowering drugs like HMG-CoA reductase inhibitors or fibrates have anti-inflammatory and maybe antiatherogenic activity by interfering with the CD40-CD154 signaling pathway [36, 37]. Antiinflammatory properties of ET receptor inhibition have been shown in cell culture models [38]. In man ET antagonists improved endothelial dysfunction, a very early phenomenon in atherogenesis [39]. So far, a therapeutic profit has been proven in patients with pulmonary arterial hypertension for the non-selective ET-A/B-receptor blocker bosentan. In pulmonary arterial hypertension, increased plasma levels of sCD154 can be found [40].

Taken together, the present data demonstrate a novel pathophysiologic link between the vasoactive peptide ET-1 and cell-cell contact mediated proinflammatory cell activation via a CD40-CD154 signaling pathway. The data further suggest that the ET system is a potential therapeutic target in cardiovascular disease.

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