A requirement for thioredoxin in redox-sensitive modulation of T-cadherin expression in endothelial cells

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INTRODUCTION

T-cad (T-cadherin), a glycosylphosphatidylinositol-anchored cadherin superfamily member, is expressed widely in the brain and cardiovascular system, and absent, decreased, or even increased, in cancers. Mechanisms controlling T-cad expression are poorly understood. The present study investigated transcriptional regulation of T-cad in ECs (endothelial cells). Conditions of oxidative stress (serum-deprivation or presence of H2O2) elevate T-cad mRNA and protein levels in ECs. Reporter gene analysis, using serially deleted T-cad promoter stretches ranging from −99 to −2304 bp, located the minimal promoter region of T-cad within −285 bp from the translation start site. Reporter activity in ECs transfected with the −285 bp construct increased under conditions of oxidative stress, and this was normalized by antioxidant N-acetylcysteine. An electrophoretic-mobility-shift assay revealed a specific nucleoprotein complex unique to −156 to −203 bp, which increased when nuclear extracts from oxidatively stressed ECs were used, suggesting the presence of redox-sensitive binding element(s). MS analysis of the nucleoprotein complex unique to −156 to −203 bp after streptavidin–agarose pull-down detected the presence of the redox-active protein thioredoxin. The presence of thioredoxin-1 in a nuclear extract from oxidatively stressed ECs was demonstrated after immunoprecipitation and immunoblotting. Transfection of ECs with thioredoxin-1 small interfering RNA abrogated oxidative-stress-induced up-regulation of T-cad transcripts and protein. We conclude that thioredoxin-1 is an important determinant of redox-sensitive transcriptional up-regulation of T-cad in ECs.

Key words: endothelial cell, oxidative stress, T-cadherin, thioredoxin, transcriptional regulation.
and demonstrated a requirement for Trx-1 (thioredoxin-1) in oxidative-stress-induced T-cad expression in ECs.

MATERIALS AND METHODS

Cell lines and cell culture

HUVECs (human umbilical-vein endothelial cells) were cultured on 0.1% (w/v) gelatin-precoated tissue-culture plates in EC growth medium containing low (2%, v/v) fetal-calf serum and EC growth supplement (PromoCell GmbH, Heidelberg, Germany) and penicillin/streptomycin (100 units/ml). The HMEC-1 (human microvascular EC) line was cultured in the same medium supplemented with 10% (v/v) fetal-calf serum. Oxidative stress in HUVECs and the HMEC-1 line was induced by culture under serum-free conditions [DMEM ( Dulbecco’s modified Eagle’s medium) containing 0.1% BSA] or by exposure to 1 mM H2O2. NAC (N-acetylcysteine; 30 mM; Sigma–Aldrich, Buchs, Switzerland) was used as an antioxidant.

In silico analysis of T-cad promoter

T-cad promoter sequence from human chromosome16 (16q24) was downloaded from the NCBI (National Center for Biotechnology Information) database. Upstream of the start site (−1 to −2304 bps) was analysed using online software to identify putative binding elements for transcription factors based on GENOMATIX (www.genomatix.de), TRANSFAC (www.cbrc.jp) and TESS (www.cbil.upenn.edu) databases.

Preparation of constructs

The T-cad promoter sequence from −1 to −2304 was cloned into pGL3 basic plasmid (Promega AG, Dübendorf, Switzerland) in between the BglII and Mlu restriction-enzyme sites. Various size deletions of promoter were generated by PCR with different primers and a fixed 3′ primer, using genomic DNA of HMEC-1 as template. The sequence of single reverse primer was 5′-TTT GTC CGA CTA GAA GGC CCC-3′ (−1 to −21) and various forward primers were, 5′-GCC AGA GCC CCT CCT CAA-3′ (−81 to −99), 5′-CCT GGT CAT CAG CCT CTA CC′ (−165 to −183), 5′-CAA ATG GGA TGC CAC CTC-3′ (−286 to −285), 5′-CGC CAG TCC CCC GGT CAA TTC-3′ (−352 to −373), 5′-GCC CCT CCC TGC CTT CTC GTG-3′ (−1109 to −1170), 5′-TGT GGG AAA CGT GAG GCT AGA TC-3′ (−1561 to −1584), 5′-GCC AGC AGA ACA GCC CAG GAA AA-3′ (−2281 to −2304). All primers were purchased from Microsynth (Balgach, Switzerland).

Transient transfection and luciferase assay

HMEC-1 cells were transfected with the various constructs using Lipofectamíne™ 2000 (Invitrogen/Molecular Probes via LuBioScience GmbH, Lucerne, Switzerland) according to the manufacturer’s protocol. A total of 105 cells were transfected with 1400 ng of either empty pGL3 plasmid or pGL3 with promoter inserts and with 100 ng of pRLTK plasmid expressing Renilla (sea pansy) luciferase to control for transfection efficiency. Firefly (Photinus pyralis) and Renilla luciferase activity were measured 48 h after transfection using a dual luciferase assay system (Promega). Firefly luminescence readings were normalized to the Renilla values.

Preparation of nuclear extract

Nuclear extract was prepared by a modified protocol formulated by Dignam et al. [32]. Subconfluent HMEC-1 cells were briefly washed with PBS and cells were collected by trypsinization. Cells were washed with PBS, followed by incubation with Buffer A (25 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT (dithiothreitol), 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM Na2VO4 and protease-inhibitor cocktail (Roche Diagnostics GmbH, Manheim, Germany)) for 30 min on ice. Nonidet P40 was added to cell suspensions (final concn. 0.1%), samples were vortexed-mixed for 10 s, then centrifuged at 17 000 g for 1 min at 4°C. Nuclear pellets were suspended in cold Buffer C (20 mM Hepes, pH 7.9, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 0.1 mM Na2VO4 and protease-inhibitor cocktail). Suspending was subjected to vigorous shaking in a rotary shaker for 1 h at 4°C and then centrifuged at 17 000 g for 10 min. Supernatants were collected, divided into portions and stored at −70°C.

EMSA (electrophoretic-mobility-shift assay)

Reactions were performed with following oligos (oligonucleotides):

Oligo −1 to −52:

5′-GTGCAAGCGCTGATGAAATGAAAAACGCCGCGG-GCCGTTCCTGACAAAAATG3′

Oligo −34 to −83:

5′-AAAGCCCTGCTCCACGGAAAATATGCTCAGTGCGAGCCGGCGTGCA TGCAAAACGAGGG-TGGCGAGCCCTCTCCTC-3′

Oligo −110 to −163:

5′-TGCTGATCTATTTGGAAGTTGGCTGGCTGGCGAGGCAGAGCCCTCTCCTC-3′

Oligo −156 to −203:

5′-GGAGGAAAATCCTGTTCCTATGATGCTGTCTGCTGTCATATTGGAAAGTGGC-3′

Oligo −194 to −243:

5′-TGTTGCTCACCCTCCCGTATCTGTGATTGAAAACGGAGGGACGGTTAGGAAAGGAT-3′

Oligo −224 to −284:

5′-CAATTGGGATGCCACCCTCCGCGCCTGCTCCTCGGCGAGGTGTTGCTCACCCTCCCG-3′

Oligos (Microsynth) were radiolabelled with [α-32P]dCTP using standard techniques. Binding reactions were performed in a 15 μl volume reaction mixture containing 1 x binding buffer (25 mM Hepes, pH 7.9, 50 mM KCl, 1 mM DTT, 1 mM EDTA and 10% glycerol), 1 μg of salmon sperm DNA, 1 μg poly(dI - dC), 1 ng of radiolabelled probe and 15 μg of nuclear extract for 30 min at 22°C. For competition assays, non-radioactively labelled oligos or non-specific DNA (100-fold the amount of labelled, unless otherwise specified) were added 15 min prior to the labelled oligos. Samples were loaded on to non-denaturing 4% (w/v) polyacrylamide gels. Gels were dried on to Whatmann paper and visualized by phosphorimaging. The following polyclonal antibodies were tested in supershift assays: anti-GATA-1 [anti-(GATA-binding protein 1)], anti-Ets1 [anti-(V-ets erythroblastosis virus E26 oncogene homologue 1)], anti-C/
EBPα [anti-(CCAAT/enhancer binding protein α)] and anti-
C/EBPβ (Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.).

**Real-time RT–PCR (reverse transcriptase–PCR)**

The procedures and conditions for real-time RT-PCR were as
described previously [12]. The primer pair specific to T-cad cDNA was:

Forward: 5′-AGA GAT GTT GGC AAG GTA GTG GA-3′
Reverse: 5′-GAA CTT GGA CCT TTC TGG CCT-3′

Primers were designed using the Primer Express software package (Applied Biosystems, Foster City, CA, U.S.A.) and obtained from Microsysynt. Gene expression was normalized with respect to the endogenous housekeeping control gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

**Biotin–streptavidin pull-down and MS analysis**

Oligo −156 to −203 (5′-GGAGGAATCCGTCTTGTAAAG-
CCATTGGTGCTCGGTCATACGCTCTACCTACATG) and its
translational oligo were biotinylated at 5′ and 3′ respectively.
Streptavidin–agarose beads (Sigma Aldrich) were pre-adsorbed
with 500 μg of BSA, 50 μg of poly(dI - dC) and 50 μg of
salmon sperm DNA for 1 h at room temperature, washed three
times with binding buffer as described above and resuspended in
300 μl of binding buffer. A 1 μg portion of annealed biotinylated
oligo was incubated with 500 μg of nuclear extract prepared
from serum-deprived HMEC-1 for 30 min at room temperature
in binding buffer as described above. For competition, non-
biotinylated oligos were incubated with nuclear extract for 15 min
before addition of biotinylated oligos. After binding reactions had
taken place, 50 μl of pre-adsorbed streptavidin–agarose beads
was added to the reaction mixture and incubation continued at
4°C for 4 h. Protein–DNA–streptavidin–agarose pull-down
complexes were processed for MS micro-sequencing by Dr Paul
Jeno (Biocenter, University of Basel, Basel, Switzerland).

**Presence of Trx in nuclear extracts and specific nucleoprotein complex**

Nuclear extract from serum-deprived HMEC-1 was immuno-
oprecipitated with anti-Trx-1 antibodies or non-immune IgG
and immunocomplexes pulled down with Protein G–Sepharose
using previously described protocols [11]. Streptavidin–agarose
pull-down was performed following incubation of nuclear
extracts from serum-deprived HMEC-1 with biotinylated oligo
−156 to −203 as detailed above. Protein G–Sepharose-bound
immunocomplexes and streptavidin–agarose-bound nucleoprotein
complexes were resolved by SDS/12 %- (w/v)-PAGE under
reducing conditions and analysed for the presence of Trx-1 by
Western blotting.

**siRNA (small interfering RNA) transfection**

Trx-1 silencing in HUVEC and HMEC-1 was performed using a previously validated siRNA oligo 5′-AUGACUGUCAG
AUGUUUGCtt [33] (lower-case letters denote dNTPs). RNA
oligo 5′-CUUCUGUUGCCUAGCAGGg was used as negative
control [11]. siRNA oligonucleotides were purchased from
Microsysynt and transfection was performed using Si-PORT Lipid
[Ambion (Europe) Ltd, Huntingdon, Cambus., U.K.) according
to the manufacturer’s recommendations. Silencing of Trx-1 was
monitored by immunoblotting at 48 and 72 h after transfection.

**Western-blot analysis**

The method of immunoblotting was described previously [10].
Cell lysis buffer was PBS containing 1% (w/v) SDS and
protease-inhibitor cocktail (Sigma). Protein concentrations were
determined using the Lowry method, and SDS/PAGE was
performed under reducing conditions. Protein loading equivalence
was routinely controlled after electrotransfer to nitrocellulose
membranes by staining with Ponceau S and by immunoblotting
for G6Pase subunit (42 kDa), β-actin (43 kDa) or GAPDH (37 kDa).
The following primary polyclonal antibodies were used: antibody
against the first extracellular domain of human recombinant T-cad
[5], anti-Trx-1 and GAPDH (Abcam, Cambridge, U.K.), anti-
(G-protein Gαs) (Calbiochem, Merck AG, Darmstadt, Germany)
and anti-β-actin (Santa Cruz Biotechnology). Secondary anti-
rabbit antibodies coupled to horseradish peroxidase were from
Southern Biotechnologies (BioReba AG, Reinach, Switzerland).
The ECL® (enhanced chemiluminescence) system (Amersham,
Rockford, IL, U.S.A.) or SuperSignal West Dura (Pierce) was
used for detection of immunoreactive proteins. Scanned images
of immunobots were analysed using AIDA Image software.
The Figures show representative images.

To quantify T-cad protein levels, precursor (130 kDa) and
mature (105 kDa) protein bands were collectively analysed.
Inter-
experimental variation in the relative intensities of the two
bands in vascular cells has been previously observed [7,10] and
was again apparent in the present study. Differential processing
of T-cad proteins has not yet been studied in any cell type.
However, we suppose that inherent differences between primary
HUVEC isolates or between HMEC-1 stock aliquots (e.g. variable
proliferation potential), or even small differences in tissue-culture
conditions (e.g. variable serum lot), may partially contribute to
inter-experimental variability.

**Cell-viability assay**

HMEC-1 cells were plated on to 96-well plates at a density of
3 × 10⁴ cells/cm², cultured in normal growth medium for 18 h,
and, after refreshing the medium, cells were incubated for 2 h
in the absence (control) or presence of different concentrations
(30 μM–1 mM) of H₂O₂. To achieve maximum lethality, cells
in a parallel plate were treated with 1 mM H₂O₂ until cell
detachment was complete (6–8 h). Fluorescence-based SYTOX™
Green inclusion assay of cell death (Invitrogen/Molecular Probes,
from LuBioScience GmbH, Lucerne, Switzerland) was performed
according to the manufacturer’s recommendations using a
fluorescence ELISA reader. The fluorescence reading under
the condition of maximal lethality was arbitrarily taken as a 100% loss of viability.

**RESULTS**

**Minimal promoter region of T-cad is localized within −285 bp**

As a first step toward functional characterization of the T-cad
promoter, we performed reporter-gene analysis with seven serially
deleted fragments of different lengths within −2304 bp from the
translation start site cloned into pGL3 vector. The first series of
reporter assays were conducted under normal serum-containing
culture conditions (Figure 1A). The shortest fragment (−99 bp)
showed insignificant levels of reporter activity, ruling out the
presence of any functional regulatory elements within −99 bp.
Transfection with the −173 bp fragment resulted in a ≈2-fold
increase in reporter activity compared with the negative control.
Maximum reporter activity, which was ≈4-fold above negative
To search for physical interactions between regulatory elements and transcription factors, we performed gel-shift assays with nuclear extracts prepared from subconfluent proliferating HMEC-1 cells and oligos designed from the T-cad promoter. Since reporter activity was observed within −173 bp and elevated in −285 bp, we designed seven different oligos starting from −1 to −284 bp; oligos were of 50–60 bp length with minimal overlap in order to maintain integrity of putative regulatory elements. The first series of gel-shift assays aimed to detect interactions between the different oligos and the nuclear extract. Apart from a number of complexes (presumably non-specific) common to all oligos, we detected a nucleoprotein complex unique to the −156 to −203 bp oligo (Figure 3A), which falls within the minimal promoter region detected by reporter-gene analysis.

To validate the specificity of this nucleoprotein complex, a competition assay was performed with different concentrations of unlabelled −156 to −203 bp oligo. We found a concentration-dependent displacement only of the complex unique to −156 to −203 bp (Figure 3B). A further competition assay compared displacement of −156 to −203 bp oligo by a non-specific oligo within the promoter (oligo −34 to −83 bp) or by unlabelled −156 to −203 bp oligo (Figure 3C). Displacement was observed only with its own unlabelled and not with the non-specific unlabelled oligo, further validating specificity of the identified nucleoprotein complex unique to −156 to −203 bp.

**The −285 bp region is modulated by oxidative stress**

Serum-deprivation-induced increase of T-cad protein in HUVECs is due to oxidative stress [10]. Having demonstrated a serum-deprivation-induced elevation of reporter activity in HMEC-1 transfected with a −285 bp construct (Figure 1), we next investigated whether −156 to −203 bp oligo might contain a redox-sensitive regulatory element.

We performed EMSA, RT–PCR and immunoblot analysis after culture of HMEC-1 cells for up to 4 h under conditions of serum deprivation. Gel-shift analysis using the −156 to −203 bp oligo revealed an increase in formation of the putative specific nucleoprotein complex within 2 h (Figure 4A), suggesting that the −156 to −203 bp oligo contains relevant binding element(s). T-cad mRNA increased steadily from ≈1.5-fold at 2 h to ≈4-fold at 4 h (Figure 4B). T-cad protein expression increased within 2 h, achieved maximum levels at ≈4 h and thereafter declined (Figure 4B), similarly to what we have previously reported using HUVECs [10].

To validate the oxidative-stress-modulated minimal promoter region of T-cad, we examined the influence of serum deprivation and H2O2 as an external source of ROS (reactive oxygen species) on the reporter activity of −285 bp. Specific ROS-dependence of the effects on reporter activity was controlled by inclusion of NAC, a well-characterized thiol-reducing agent that augments the intracellular glutathione pool and counteracts the actions of ROS. Both serum deprivation and H2O2 treatment of HMEC-1 cells transfected with the −285 bp construct increased reporter activity 2-fold above that under control serum-containing conditions, and these oxidative-stress inductions of reporter activity were abrogated in the presence of NAC (Figure 5A). These results suggest that regulatory element(s) located in −285 bp might be responsible for redox-sensitive modulation of T-cad.

Transcription-factor(s)-binding element is present on −156 to −203 bp

**Figure 1** Reporter-gene analysis for the T-cad promoter

HMEC-1 cells were co-transfected with 1400 ng of various deletion fragments cloned in pGL3 and 100 ng of pRLTK (internal control). Luciferase activity was measured in cells cultured under normal serum-containing culture conditions (A) and following 3 h of serum-deprivation (B). Results show luciferase activity relative to a negative control (empty pGL3) and represent means ± S.D. for at least four independent experiments. The asterisk indicates significant difference (*P < 0.01*) between serum-present (Serum) and serum-deprivation (Serum-free) conditions.

Promoter structure of T-cad

The sequence of the T-cad minimal promoter region detected by reporter-gene analysis is presented in Figure 2. Previous studies had demonstrated that T-cad promoter is TATA-less and a GC-box-like sequence is located at −266 bp [18,28]. Additionally, three possible transcription start sites at different locations, −73, −120 and −473 bp, in malignant cells have been identified [18,28]. Database-predicted consensus sequences of regulatory elements are shown in Figure 2.
Regulation of T-cadherin expression by thioredoxin

The promoter region of the T-cad gene was analysed using various databases (see the Materials and methods section). The asterisk (*) denotes previously reported transcription start sites [18,28]. The large arrowhead (A) denotes the start codon. In silico-predicted regulatory elements are indicated in bold font, oriented with arrows and labelled below the sequence. A GC-box-like sequence is located at −266. Ets-transcription-factor-binding elements (GGAA/T) were predicted at −277, −278, −295, −315, −335, and −365 bp. C/EBP-binding elements (CCAAT) were predicted at −529 and −49 bp. An AHR element (GCGTG) was predicted at −139 bp. The −126 to −203 bp stretch is shaded; previously reported [28] putative binding sites for ER (GGTCA) and GR (CAGC) within this stretch are indicated by braces and labelled above the sequence.

Figure 2 5′ flanking region of the T-cad gene

Figure 3 EMSA reveals formation of a specific nucleoprotein complex within −156 to −203 bp

(A) Gel-shift assays were performed with radiolabelled oligos designed from the T-cad promoter from −1 to −284 bp and nuclear extracts of proliferating subconfluent HMEC-1 cells. Samples were resolved on 4% (w/v) polyacrylamide gels, and nucleoprotein complexes were detected by phosphorimaging. A representative image from three separate experiments is presented. Apart from complexes common to all oligos and presumed non-specific (n.s.), a complex unique to the −156 to −203 bp oligo was observed (indicated by the arrow). Differences in intensity of assumed non-specific complexes are due to non-uniform labelling. A competition assay was performed using radiolabelled −156 to −203 bp oligo with various amounts (denoted by [x]) of non-radiolabelled ('cold') −156 to −94 bp oligo (B) or with either non-radiolabelled −156 to −203 bp oligo or non-specific −34 to −83 bp oligo, each at 100 × [x] (C). The double-headed arrow indicates the specific nucleoprotein complex.

increase in T-cad transcripts and T-cad protein respectively, confirming redox-sensitive modulation of T-cad expression (Figure 5B). Analysis of dose-dependent effects of H2O2 on the expression of T-cad transcript and protein expression and viability (assessed with SYTOX Green staining) in HMEC-1 cells showed up-regulation of T-cad expression also at lower doses of H2O2 (≥50 μM) (see Supplementary Figure S1 at http://www. BiochemJ.org/bj/416/bj4160271add.htm). Furthermore, and as shown previously for the time-dependent effects of serum deprivation on T-cad expression [10], up-regulation of T-cad in response to H2O2 was associated with the onset of apoptosis.

T-cad induction under oxidative stress is Trx-dependent

We attempted to identify directly transcription factor(s) within the nucleoprotein complex unique to −156 to −203 bp using different approaches. The relevance of in silico predicted
regulatory elements within −156 to −203 bp (Figure 2; shaded region) was investigated by using a supershift assay. Antibodies tested included anti-C/EBPα, anti-C/EBPβ, anti-Ets1/2 and anti-GATA-1, but in no case was either a supershift or squelching of specific nucleoprotein complex detected (results not shown). Several efforts to identify transcription factors in nucleoprotein complexes specific to the −156 to −206 bp oligo by excision after EMSA and microsequencing also failed. A third approach was to incubate nuclear extracts from serum-deprived HMEC-1 with biotinylated −156 to −203 bp in the absence and presence of non-biotinylated −156 to −203 bp as competitor, and perform streptavidin–agarose pull-down followed by microsequencing. Microsequencing of complexes pulled down failed to identify a known transcription factor, but did reveal the presence of a peptide with homology with Trx in the complex pulled down from nuclear extracts incubated with biotinylated −156 to −203 bp and without competitor. Trxs are members of a class of small (≈12 kDa) redox-active proteins; Trx-1 is normally a cytosolic protein, but certain stimuli, including oxidative stress, induce its translocation to the nucleus, where it can modulate transcription-factor activities [34]. Western-blot analysis of the complexes confirmed the presence of Trx-1 in a complex pulled down from the biotinylated −156 to −203 bp/nuclear extract incubation mixture; Trx-1 was absent when non-biotinylated −156 to −203 bp oligo was included as a competitor during incubations (Figure 6A). The presence of Trx-1 in nuclear extracts from serum-deprived HMEC-1 cells was additionally demonstrated by direct immunoprecipitation and subsequent Western-blot analysis; Trx-1 was present in anti-Trx-1 antibody precipitates, but not in non-immune antibody precipitates (Figure 6B).

To investigate the functional involvement of Trx-1 in the control of T-cad expression under conditions of oxidative stress, we exploited siRNA to knock down Trx-1 expression in both HMEC-1 cells and HUVECs. In both EC types, transfection with Trx-1-siRNA decreased Trx-1 protein levels by ±60% within 48 h, which persisted at 72 h (Figure 6C). Trx-1 levels in HMEC-1 cells or HUVECs were not affected by transfection with control (non-specific) siRNA. Transfection of HMEC-1 cells or HUVECs with Trx-1-specific siRNA abrogated serum-deprivation (Figure 7A or 8A respectively) and H2O2 (Figure 7B or 8B respectively)-induced increases in T-cad protein expression. Positive regulation of T-cad gene expression in HMEC-1 cells and HUVECs by Trx-1 under conditions of oxidative stress was confirmed by RT–PCR analysis; Trx-1 siRNA prevented serum-deprivation- and H2O2- induction of T-cad mRNA in HMEC-1 and HUVEC (Figures 7C and 8C respectively). Knockdown of Trx-1 abrogated the induction of T-cad mRNA and protein in response to lower doses (50 and 100 μM) of H2O2 (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/416/bj4160271add.htm). These results support the notion that Trx-1 is required for up-regulation of T-cad under conditions of oxidative stress in ECs.
Regulation of T-cadherin expression by thioredoxin

Figure 6 Trx is present in a nucleoprotein complex specific to −156 to −203 bp oligo

(A) Streptavidin-agarose pull-down was performed after incubation of nuclear extracts from serum-deprived HMEC-1 cells with biotinylated −156 to −203 bp in the absence (−) or presence (+) of non-biotinylated −156 to −203 bp as competitor. Bound nucleoprotein complexes were analysed by Western blotting for the presence of Trx-1. (B) Nuclear extracts from serum-deprived HMEC-1 cells were immunoprecipitated with anti-Trx-1 antibodies and non-immune (n/i) IgG. Immunocomplexes were analysed by Western blotting for the presence of Trx-1 (12 kDa). (C) HMEC-1 cells and HUVECs were transduced with Trx-1-siRNA (Trx-1-si) or control siRNA (C-si) and, after 48 and 72 h, whole-cell lysates were immunoblotted for Trx-1 and GAPDH.

DISCUSSION

The present study investigated transcriptional regulation of T-cad in ECs. The main findings are:

(1) the minimal promoter region of T-cad is located within −285 bp from the translation start site

(2) (a) transcription factor(s) binding regulatory element responsible for oxidative stress-induced T-cad up-regulation is (are) present between −156 and −206 bp from the translation start site

(3) Trx-1 protein is important for the up-regulation of T-cad expression in ECs under conditions of oxidative stress.

In contrast with CArG [CC(A + T-rich)6GG] boxes as muscle-specific regulatory elements [35], EC-specific regulatory elements have not been reported. Spatial and temporal differences in endothelial gene expression have been proposed to account for endothelial heterogeneity and vascular diversity [36,37]. Similarities exist between promoter of T-cad and those of other cadherins and endothelial-specific genes. For example, the T-cad promoter is TATA-less [18,28], as are E-cadherin [38], P-cad [39] and endothelial markers such as eNOS (endothelial nitric oxide synthase) [40] and VEGF (vascular endothelial growth factor) [41]. Transcription start sites at three different positions, namely at −73, −120 and −473 bp, have been located in osteosarcoma cells [28] and in lung-cancer cell lines [18]. We observed minimal promoter activity within −285 bp, but not within −99 bp, indicating that the −120 bp transcription start site might be functional in ECs. A previous study putatively correlated location of an AHR element at −45 bp with Ahrgan-dependent repression of T-cad in rat smooth-muscle cells [29]. Since the −99 bp construct did not exhibit any reporter activity in ECs, it is possible that differences in regulation of T-cad within different constituent cells of the vasculature exist. Support for this assumption derives from observations that vasoactive substances, such as angiotensin II and thrombin, induce T-cad expression in human vascular smooth-muscle cells but not in ECs (M. B. Joshi and T. J. Resink, unpublished work).

Figure 7 Trx is a regulator of T-cad expression in HMEC-1 cells under conditions of oxidative stress

At 72 h after transduction of HMEC-1 cells with Trx-1-siRNA (grey bars) or control siRNA (C-siRNA; white bars) cells were deprived of serum for 4 h (A and C) or treated with 1 mM H2O2 for 2 h (B and C). Whole-cell lysates were immunoblotted for T-cad, Trx-1 and GAPDH (A and B) and T-cad mRNA was quantified by RT–PCR with normalization to the GAPDH gene (C). Protein and transcript levels are expressed relative to values in control siRNA-transduced HMEC-1 cells under normal serum conditions. Results are means ± S.D. for three independent experiments (*P < 0.01).

In the present study, ECs transfected with −285 bp exhibited a 4-fold increase in minimal reporter activity, whereas a 9-fold increase in reporter activity has been demonstrated in osteosarcoma cells transfected with −434 bp [28]. Additionally the minimal reporter activity observed within −285 bp in ECs remained steady with all further deletion fragments tested. An abundance of transcription factor(s), different regulatory proteins or malignancy might explain the higher reporter activity in osteosarcoma cells. Our findings that the antioxidant NAC abrogated oxidative-stress-induced reporter activity in ECs transfected with −285 bp, and that specific nucleoprotein complex formation (using −156 to −203 bp oligo) was greater in extracts from oxidatively stressed ECs, suggest the presence of a redox-modulated regulatory element present within...
Binding element), one GATA and two copies of CCAAT (C/EBP-binding element) are located. We were unable to identify a relevant transcription factor either by gel-shift assays using antibodies against Ets1/2, GATA or C/EBP, or by excision and microsequencing of the nucleoprotein complex after EMSA. However, microsequencing of nucleoprotein complex after use of biotinylated −156 to −203 bp oligo and streptavidin–agarose pull-down indicated the putative presence of Trx. Western-blot analysis confirmed the presence of a 12 kDa Trx-1 protein within the nucleoprotein complex unique to the −156 to −203 bp oligo. The presence of Trx-1 protein in nuclear extracts from serum-deprived HMEC-1 cells was further demonstrated by direct immunoprecipitation with anti-Trx-1 antibodies. These data are in accordance with those obtained in a recent study demonstrating nuclear import of Trx-1 in ECs and cardiomyocytes subjected to oxidative stress [42].

Trxs compose a class of small multifunctional 12 kDa redox-active proteins that maintain the reductive intracellular potential. Trx participates in redox reactions by oxidation of its active-site thiol groups and is then reduced by NADPH in a reaction catalysed by the flavoenzyme Trx reductase. Apart from its oxidoreductase functions, Trx controls the activity of a variety of target proteins containing regulatory cysteine residues via reversible thiol–disulfide exchange reactions [34,43–45]. Another regulatory mechanism independent of thiol redox activity depends upon the ability of Trx to interact with other proteins to form stable functional protein complexes [45]. These pleiotropic functions of Trx affect diverse cellular processes, including gene expression, signal transduction, proliferation and apoptosis [34,43–45].

Cells are equipped with two Trx species: Trx-1 is primarily present in the cytosol, whereas Trx-2 is located in mitochondria. Several stimuli, including oxidative stress, induce Trx-1 to translocate from the cytoplasm to the nucleus, where it regulates the DNA-binding activity of redox-sensitive transcription factors containing critical cysteine residues in their DNA-binding domains, including NF-κB (nuclear factor-κB), GR, ER, HIF-1α (hypoxia-inducible factor 1α), AP-1 (activator protein 1) and p53 [34,43–45]. Trx-1 affects the activity of its target transcription factors by either direct dithiol–disulfide exchange interactions (e.g. NF-κB, GR and ER) and/or indirectly (e.g. NF-κB, AP-1 and p53) via binding to another nuclear redox protein redox factor 1 [34,43–45]. Trx may also interact with transcription factors by mechanisms that do not directly depend on dithiol–disulfide exchange: transcription regulators NusG (N-utilityization substance G protein) and RcsB (regulator of capsule synthesis B component), which do not contain regulatory cysteine residues, were found in Trx complexes isolated from Escherichia coli, and these interactions were stable in the presence of reducing agents [45].

We did not identify a target transcription factor with which Trx-1 might interact to induce T-cad transcript expression in ECs. We also do not know which regulatory element within −156 to −203 bp of the T-cad gene might be involved; this region does not contain the antioxidant-responsive element that can bind Trx-regulated transcription factors. However, we demonstrated functional relevance for Trx-1 in regulating T-cad expression. siRNA-mediated knockdown of Trx-1 protein in either HMEC-1 cells or HUVECs resulted in an abrogation of serum-deprivation-induced and H₂O₂-induced T-cad mRNA and protein expression. These data show that Trx-1 is necessary for T-cad transcription activation in ECs under conditions of oxidative stress. The mechanism whereby Trx-1 might affect T-cad transcription is unknown. Antioxidant functions of Trx-1 are unlikely to be involved, since Trx-1 knockdown abrogated, rather than enhanced, oxidative-stress-induced T-cad transcript expression. Possibly Trx-1 acts more directly [e.g. as an auxiliary

Figure 8 Trx is a regulator of T-cad expression in HUVECs under conditions of oxidative stress

At 72 h after transduction of HUVECs with Trx-1-siRNA (grey bars) or control siRNA (CsiRNA; white bars) cells were deprived of serum for 4 h (A and C) or treated with 1 mM H₂O₂ for 2 h (B and C). Whole-cell lysates were immunoblotted for T-cad, Trx-1 and β-actin/GAPDH (A and B), and T-cad mRNA was quantified by RT–PCR with normalization to the GAPDH gene (C). Protein and transcript levels are expressed relative to values in control siRNA-transduced HUVECs under normal serum conditions. Results are means ± S.D. for three independent experiments (**P < 0.01).
transcription (co)factor] in the process of T-cad transcription regulation during oxidative stress.

Trx-1 is a recognized inhibitor of oxidative-stress-induced apoptosis in different cell types, including ECs [42], although the mechanisms for the anti-apoptotic effects of Trx-1 are very poorly understood. Binding of cytosolic Trx-1 to ASK1 (apoptosis signal-regulating kinase 1) renders it inactive and prevents ASK1-induced apoptosis ([34,44]). Glutathione transferase P1 has been identified as a downstream target for Trx-1-dependent anti-apoptosis effects during oxidative stress; nuclear import of Trx-1 and activation of the glutathione transferase P1 gene was shown to be essential for apoptosis inhibition [42]. Up-regulation of T-cad protects EC from oxidative-stress-induced apoptosis [10]. We suggest that Trx-1-dependent activation of T-cad gene expression during oxidative stress could be a novel mechanism for the anti-apoptotic effects of Trx-1.

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REFERENCES
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SUPPLEMENTARY ONLINE DATA

A requirement for thioredoxin in redox-sensitive modulation of T-cadherin expression in endothelial cells

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Figure S1 Dose-dependent effects of H2O2 on cell viability and T-cad expression in HMEC-1 cells

HMEC-1 cells were incubated for 2 h in the absence (control) or presence of the indicated concentration of H2O2. Cell viability was determined by the fluorescence-based assay of SYTOX Green inclusion (A). Values for SYTOX Green inclusion are expressed relative to that in cells which had been treated with 1 mM H2O2 for 8 h to induce maximum cell death (i.e. maximum staining). T-cad mRNA was quantified by RT–PCR with normalization to the GAPDH gene (B). T-cad protein was estimated by immunoblotting (C). Transcript and protein levels are expressed relative to the value in the control (taken as 1). Results in (A) and (B) represent means±S.D. for three independent experiments. Up-regulation of T-cad protein at the lower doses of H2O2 (50 and 100 μM) was confirmed in three other experiments (see Supplementary Figure S2).

Figure S2 Knockdown of Trx-1 prevents induction of T-cad transcript expression in HMEC-1 cells at lower doses of H2O2

At 72 h after transduction of HMEC-1 cells with Trx-1-siRNA (grey bars) or control siRNA (C-siRNA; white bars) cells were treated with the indicated concentrations of H2O2 for 2 h. T-cad mRNA was quantified by RT–PCR with normalization to the GAPDH gene (A). Whole-cell lysates were immunoblotted for T-cad, Trx-1 and GAPDH (B). Protein and transcript levels are expressed relative to values in C-siRNA-transduced HUVECs under normal serum conditions. Results are means±S.D. for three independent experiments (**P < 0.01).

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