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Effects of Antisense Oligonucleotide-Mediated Depletion of Tumor Necrosis Factor (TNF) Receptor 1-Associated Death Domain Protein on TNF-Induced Gene Expression

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ABSTRACT

Tumor necrosis factor (TNF) receptor 1-associated death domain protein (TRADD) is an adaptor protein known to be involved in the TNF signaling pathway as well as signaling of other members of the TNF receptor superfamily, including DR3, DR6, p75^{NTR}, and the Epstein-Barr virus latent membrane protein 1. Current knowledge of the function of the adaptor protein has been derived from studies examining its over-expression in either wild-type or mutated forms. In this study, we analyzed the consequences of antisense oligonucleotide (ASO)-mediated depletion of endogenous TRADD on TNF induction of inflammation-related gene products, such as intercellular adhesion molecule-1, and associated kinase signaling pathways in human umbilical vein endothelial cells. A broader perspective of TRADD's role in TNF signaling was indicated by microarray gene expression analysis, where 20 of 24 genes that showed a 5-fold or greater increase in TNF-induced mRNA expression levels displayed a reduction in TNF-induced expression as a consequence of ASO-mediated knockdown of TRADD. Reduced activation of the nuclear factor-κB and c-Jun NH_2-terminal kinase pathways, as measured by IκB-α protein levels and the extent of c-Jun phosphorylation, was also observed. These results indicate usage of antisense inhibitors of TRADD expression for modulating diseases associated with TRADD-dependent signal transduction pathways.

Tumor necrosis factor (TNF) is a pleiotropic cytokine whose overproduction has been implicated in the development and progression of many inflammatory, infectious, and autoimmune diseases (Beutler, 1999). Most cellular responses to TNF, including activation of second messenger systems and changes in gene expression levels, have been attributed to the interaction of TNF with tumor necrosis factor receptor 1 (TNF-R1), (Slowik et al., 1993; MacEwan, 2002; McFarlane et al., 2002). Binding of TNF to the extracellular domains of TNF-R1 promotes release of the silencer of death domains protein from its association with the intracellular death domain of the receptor (Jiang et al., 1999). Release of silencer of death domains from the receptor's death domain allows for binding of the 34 kDa TNF-R1-associated death domain protein (TRADD), which contains a death domain in its C-terminal region. TRADD is expressed both constitutively and ubiquitously (Hsu et al., 1995).

TNF-induced association of TRADD with the death domain of TNF-R1 leads to recruitment of other receptor adapter proteins [e.g., TNF receptor-associated factor 2 (TRAF2) (Hsu et al., 1996a) and receptor interacting protein (RIP) (Hsu et al., 1996b)] to form a membrane-associated signaling complex (sometimes referred to as a signalsome). Once assembled, the TNF-R1 signaling complex initiates a spectrum of kinase-mediated phosphorylation events that leads to activation of gene expression at the transcription level (Introna and Mantovani, 1997; Madge and Pober, 2001; MacEwan, 2002). The majority of genes whose expression is induced by TNF are regulated by the activation protein 1 (AP-1) and

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ABBREVIATIONS: TNF, tumor necrosis factor; TNF-R1, tumor necrosis factor receptor 1; TRAF2, TNF receptor associated factor 2; TRADD, TNF receptor 1 associated death domain protein; RIP, receptor interacting protein; AP-1, activation protein 1; NF-κB, nuclear factor κB; SODD, silencer of death domains; FADD, Fas-associated death domain protein; ASO, antisense oligonucleotide; 2′-MOE, 2′-*O*-(2-methoxyethyl); HUVEC, human umbilical vein endothelial cell; IL-1β, interleukin 1β; RT-PCR, quantitative real-time polymerase chain reaction; ICAM-1, intercellular adhesion molecule 1; MM, mismatch; NT, no treatment; VCAM, vascular cell adhesion molecule; GM-CSF, granulocyte macrophage-colony-stimulating factor; JNK, c-Jun NH_2-terminal kinase; LMP1, latent membrane protein 1; AP-1, activation protein 1; TNF-R2, tumor necrosis factor receptor 2; Fas, Fas antigen; 6-FAM, 6-carboxyfluorescein; TAMRA, 5-carboxytetramethylrhodamine.

nuclear factor kappa B (NF-κB) transcription pathways. These pathways have been shown to control a number of aspects of the inflammatory response, including increased expression of cell adhesion molecules, prostaglandins, and chemokines by the vascular endothelium (Introna and Mantovani, 1997; Madge and Pober, 2001).

By virtue of its death domain, TRADD also has the ability to bind Fas-associated death domain protein (FADD) that in turn initiates programmed cell death by binding either the caspase inhibitory protein c-FLIP or procaspase-8. TRADD associates with a number of other death domain-containing receptors, including DR3 (Chinnaiyan et al., 1996), DR6 (Pan et al., 1998), and $p75^{NTR}$ (Cantarella et al., 2002), all members of the TNF receptor superfamily. Thus, TRADD is indicated as specific for inflammatory and death-inducing responses mediated by TNF and certain TNF-related ligands.

Because of its direct association with TNF-R1 and other receptor adaptor proteins, TRADD is thought to play a pivotal role in a number of TNF-mediated responses. This perception has largely been supported by overexpression of wild type and dominant-negative mutant forms of TRADD in cultured cells (Hsu et al., 1996a; Park and Baichwal, 1996). The abnormally high expression levels of TRADD and its mutants as elicited by this approach, however, lend a certain degree of uncertainty in defining its function solely in this manner (Koller et al., 2000). Abnormally high expression levels may lead to alterations in the distribution and binding equilibriums of the protein to result in protein-protein interaction artifacts. Furthermore, determination of the physiological role of TRADD and phenotype in the whole organism, as well as its function(s) in differentiated cell types has yet to be achieved.

In this report, we have evaluated TRADD's role in TNF signaling by knockdown of the endogenous protein with ISIS 25291, an antisense oligonucleotide (ASO) inhibitor of TRADD expression. ASOs are short, synthetic oligonucleotides, generally 15 to 25 nucleotides in length, designed to inhibit expression of a target protein by sequence-specific hybridization to its respective mRNA through Watson-Crick base-pair interactions. In the past decade, ASOs have demonstrated efficacy in the therapeutic treatment of human diseases (Bennett, 1999) and have proven their utility in the dissection of gene function and validation of gene targets in vitro (Bennett, 1999; Koller et al., 2000) and in vivo (Zhang et al., 2000). Improvements in ASO potency and duration of action (McKay et al., 1999; Zhang et al., 2000) have resulted from modifications of the phosphodiester backbone, sugar, and bases (Cook, 1998). The 2′-*O*-(2-methoxyethyl) modified sugar residue (2′-MOE) has been of particular interest because of the high degree of nuclease resistance and target RNA affinity (Cook, 1998; McKay et al., 1999) that the modification imparts to an oligonucleotide. ISIS 25291 is a 2′-MOE modified oligodeoxynucleotide that has been designed to reduce TRADD expression via RNase H-mediated degradation of the mRNA.

Materials and Methods

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were obtained either from commercial sources (Clonetics, Walkersville, MD, or Cascade Biologicals, Portland, OR) and cultured in growth medium as recommended by the suppliers or isolated directly from human umbilical vein and cultured as described previously (Madge and Pober, 2000). Cells were maintained in a humidified chamber with a constant temperature of 37°C and 5% CO_2. For cytokine induction, either 5 ng/ml TNF or 10 ng/ml IL-1β (R&D Systems, Minneapolis, MN) was used for the times indicated in Figs. 1, 2, and 4 and Table 3 before cell harvest.

Oligonucleotide Synthesis. Oligonucleotides were synthesized and purified as described previously (Sanghvi et al., 1999). Oligonucleotide sequences and compositions are described in Table 1.

Oligonucleotide Treatment. The day before transfection, cells were plated at a density such that they were less than 50% confluent at the time of transfection. Transfection mixes were assembled by combining Lipofectin (Invitrogen, Carlsbad, CA) with the indicated molarity of oligonucleotide in OptiMEM (Invitrogen) such that the final lipid concentration was either 3 μg/ml per 100 nM oligonucleotide. Transfection mixes were preincubated at room temperature for 30 min to facilitate complex formation before their application to the cells. Normal growth media were removed, and cells were washed with Opti-MEM before addition of transfection mixes. Cells were incubated at 37°C, 5% CO_2, for 4 h in the presence of the transfection mix before media exchange with normal growth medium. Cells were allowed to continue growth for the indicated times before addition of cytokine and/or harvest.

Quantitative RT-PCR. mRNA levels were measured by the quantitative real-time polymerase chain reaction (RT-PCR) method (Winer et al., 1999). Total RNA was isolated using a RNeasy Mini prep kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Five to ten nanograms of total RNA was combined with 100 nM concentrations of each of the gene-specific dual-labeled probes, and forward and reverse primers in a buffered solution consisting of 1× TaqMan buffer A (Applied Biosystems, Foster City, CA), 5.5 mM $MgCl_2$, 300 μM concentrations of each dNTP (Amersham Biosciences, Piscataway, NJ), 2 units of RNase inhibitor, 0.625 units of AmpliTaq Gold, and 6.25 units of murine leukemia virus RT. Except for dNTP solutions, all reagents above were obtained from Applied Biosystems. Quantitative RT-PCR reactions were conducted and analyzed on the ABI Prism 7700 sequence detector (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase mRNA levels were used as the internal reference for normalization between samples. Primer probe set sequences (5′→3′) for each transcript are as shown in Table 2.

Western Blot. Total cellular protein was harvested in cell lysis buffer composed of phosphate-buffered saline, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor tablets (Roche Diagnostics, Indianapolis, IN). Protein concentrations were determined using the Bio-Rad detergent-compatible protein assay kit (Pierce Biotechnology, Rockford, IL). Equal quantities of each protein sample were precipitated with 8 volumes of acetone at −80°C for 1 h or −20°C overnight, and protein pellets were resuspended in sample load buffer (Invitrogen, Carlsbad, CA) containing 5% β-mercaptoethanol. Samples were heated at 95°C for 5 min and subsequently separated on a 10% Tris-glycine gel (Invitrogen)

TABLE 1

Sequence and composition of ISIS 25291 and mismatch control oligonucleotides

Each oligonucleotide is composed of a 10-base phosphorothioate modified 2′deoxy domain positioned in the center, flanked by two 4-base 2′-MOE phosphodiester modified domains on the 5′ and 3′ ends. 2′-MOE modified nucleotides are indicated by underline, all cytosine residues are methylated at the five position, and mismatch bases are indicated in bold.

Oligonucleotide	Mismatches	Sequence (5′→3′)
ISIS 25291	0	GCTCGTACTCGTAGGCCA
ISIS 25290	5	GCACGAACTCTTAAGCTA
ISIS 100720	4	GCTCGCATTCATGGGCCA
ISIS 110731	6	GCACCTAGTGGTTGCCCA
ISIS 110732	8	GCACCTAGTGGATGCCCT

and transferred to a polyvinylidene difluoride membrane. TRADD and FADD antibodies (BD Transduction Laboratories, Lexington, KY) were used at 1:1000 dilution. β-Actin antibody (Sigma, St. Louis, MO) was used at 1:5000 dilution. Horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) were used at 1:2500 dilution. ECL+ chemiluminescent detection system (Amersham Biosciences) was employed for visualization of protein bands. For the determination of phosphorylation state, total cellular protein was harvested in cell lysis buffer containing phosphatase inhibitors (Cell Signaling Technology, Beverly, MA). Phosphospecific antibodies and IκB-α antibody (1:1000 dilution) were obtained from Cell Signaling Technology and applied in accordance with the manufacturer's protocol unless otherwise indicated.

Flow Cytometry. Cells were harvested and stained with phycoerythrin-labeled anti-ICAM-1 antibody (Ancell, Bayport, MN) in cell staining buffer (phosphate-buffered saline containing 2% BSA and 0.2% sodium azide) for 1 h. Cells were washed once in cell staining buffer and resuspended in FACSFlow buffer containing 0.5% formaldehyde and analyzed on an BD Biosciences FACScan to determine mean fluorescence intensity. Phycoerythrin-labeled isotype control was used for staining untreated cells to ascertain background fluorescence.

Affymetrix GeneChip Sample Preparation. GeneChip hybridization samples were prepared from total RNA as described by the manufacturer (Affymetrix Inc., Santa Clara, CA). In brief, double-stranded cDNA was synthesized from isolated total RNA (RNeasy MiniKit; QIAGEN) using a chimeric oligodeoxynucleotide primer (Integrated DNA Technologies, Coralville, IA) composed of $(dT)_{24}$ at the 3′ end for priming the first-strand cDNA synthesis by Superscript II reverse transcriptase (Invitrogen) and the T7 RNA polymerase promoter sequence at the 5′ end for synthesis of the biotin-labeled cRNA using the Enzo BioArray high-yield RNA transcript labeling kit (Affymetrix). Labeled cRNA was purified then fragmented into lengths of 35 to 200 nucleotides by heat denaturation in the presence of magnesium. Integrity of the total RNA and biotin-labeled cRNA product was confirmed by agarose gel electrophoresis, using the 18S and 28S rRNAs as indicators for total RNA integrity and the range of full-length cRNAs as the indicator for cRNA integrity. Fragmented biotin-labeled cRNA samples were then hybridized to the U95Av2 GeneChip at 45°C for 20 h. Hybridized chips were washed and double-stained using the Fluidics Station 400 (Affymetrix) as defined by the manufacturer's protocol.

Affymetrix GeneChip Analysis. Stained GeneChips were scanned for probe cell intensity with the GeneArray scanner (Affymetrix). Probe cell intensity values (minus average background intensity) were normalized by a factor of 1 to the global array signal, using a target average global intensity of 2500. Signal values for each probe set were calculated using Affymetrix Microarray Suite v5.0 software. Each condition was profiled from biological duplicates, one chip per sample. Fold change was computed using the geometric mean of signal values as generated by MASv5. Signal values were log-transformed with base 2 for statistical analyses of the data set. Statistical tests were performed using SAS and S-Plus as follows. One-way analysis of variance was performed first. The least-significant-difference t test was then conducted to compare specific groups of interest after one-way analysis of variance. Significant genes were identified using the two-tailed t test with the criterion of $p \leq 0.05$. Agglomerative hierarchical clustering (Eisen et al., 1998) was performed on the basis of expression profiles of eight GeneChip samples to investigate relationships between samples. Complete linkage was adopted and one minus the Pearson correlation coefficient was used as a dissimilarity measure.

TABLE 2

Primers and probe sets

Forward primers (FP), reverse primers (RP), and dual-labeled probes (T) used for real-time RT-PCR analysis of listed mRNAs. GenBank accession numbers for each sequence are in parentheses.

TRADD (NM_003789)	
FP	ACGAGGAGCGCTGTTTGAGT
RP	TCCAGCTCAGCCAGTTCTTCAT
T	6FAM-CCAGCAGCCCGACCGGCTC-TAMRA
ICAM-1 (NM_000201)	
FP	CATAGAGACCCCGTTGCCTAAA
RP	TGGCTATCTTCTTGCACATTGC
T	6FAM-CTCCTGCCTGGGAACAACCGGAA-TAMRA
VCAM-1 (NM_001078)	
FP	GGGAAGCCGATCACAGTCAA
RP	ATGAGATGATCTCCTTTCAGTAAGTCTATC
T	6FAM-TCAGTTGCTGATGTATACCCATTTGACAGGC-TAMRA
IL8 (NM_000584)	
FP	GAAGGAACCATCTCACTGTGTGTAA
RP	AAATCAGGAAGGCTGCCAAGA
T	6FAM-CATGACTTCCAAGCTGGCCGTGG-TAMRA
E-selectin (NM_000450)	
FP	AATGTGTGGGTCTGGGTAGGAA
RP	TCCACGCAGTCCTCATCTTTT
T	6FAM-CTGGGCTCCAGGTGAACCCAACA-TAMRA
CSF2 (NM_000758)	
FP	CCCTGGGAGCATGTGAATG
RP	CAGCAGTGTCTCTACTCAGGTTCAG
T	6FAM-ATCCAGGAGGCCCGGCGTCT-TAMRA
TNFAIP3 (NM_006290)	
FP	CTGCCCAGGAATGCTACAGATAC
RP	CAGGGTCACCAAGGGTACAAA,
T	6FAM-CCATTGTTCTCGGCTATGACAGCCATC-TAMRA
IRF1 (NM_002198)	
FP	CATGGCTGGGACATCAACAAG
RP	GCTTTGTATCGGCCTGTGTGA
T	6FAM-ATGCCTGTTTGTTCCGGAGCTGGG-TAMRA
CD69 (NM_001781)	
FP	CCATTTCTCAACACGTCATGA
RP	AATGGTGATGAAGACCACAT
T	6FAM-CCTTCCAAGTTCCTGTCCTGTGTGC-TAMRA
G3PDH (NM_002046)	
FP	GAAGGTGAAGGTCGGAGTC
RP	GAAGATGGTGATGGGATTTC
T	6FAM-CAAGCTTCCCGTTCTCAGCC-TAMRA

Results

ISIS 25291 Promotes a Dose-Dependent Reduction of TRADD Protein and Subsequent Reduction in TNF-Induced ICAM-1 Expression. ISIS 25291 is an 18-base modified oligonucleotide that complements a region located in exon 5 of the human TRADD gene. ISIS 25291 and its mismatch sequence controls (Table 1), are each composed of four 2′-MOE nucleosides at both the 5′ and 3′ ends and 10 contiguous 2′-deoxy phosphorothioate nucleosides positioned in the center. The 2′-MOE modified domains increase the binding affinity for the target RNA, and provide additional resistance to exonuclease activity (McKay et al., 1999). The central domain produces a suitable substrate for endogenous RNase H once hybridized to the complementary target RNA (Wu et al., 1999).

TRADD protein levels were dramatically reduced in HUVECs treated with ISIS 25291 and subsequently cultured for 48 h to allow pre-existing TRADD protein to be cleared through normal degradative processes (Fig. 1A). The magnitude of TRADD mRNA and protein reductions in HUVECs was dependent on the extent of the oligonucleotide's sequence complementarity to the target site within the TRADD transcript. An increase in the number of base mismatches correlated with a loss of antisense oligonucleotide activity, as indicated by a decrease in the effect of oligonucleotide treatment on TRADD mRNA and protein levels with an increase in number of mismatches.

A sequence-dependent decrease of TNF-induced expression

of intercellular adhesion molecule 1 (ICAM-1) was also observed in HUVECs treated with ISIS 25291 compared with the respective mismatch controls (Fig. 1B). Furthermore, ISIS 25291 caused a dose- and sequence-dependent decrease in induction of ICAM-1 by TNF but not by IL-1β (Fig. 2). The close similarity between reductions in TRADD protein level and subsequent ICAM-1 induction highlights TRADD's role in TNF-induced ICAM-1 expression.

High-Density DNA Array Gene Expression Profiles of TNF-Induced HUVECs Treated with ISIS 25291. Additional genes involved in TNF signaling were identified through usage of the Affymetrix DNA microarray technology. HUVECs were treated with 100 nM ISIS 25291 (ASO) or the respective 8-base mismatch control oligonucleotide (8MM), ISIS 110732, and subsequently induced with TNF 68 h after transfection. Hierarchical clustering of the array data set, with complete linkage, showed that TNF stimulation induces large changes in gene expression compared with basal, and that ISIS 25291 treatment modulates the gene expression profile such that it more closely resembles that of basal levels (Fig. 3). The order in which clusters are joined suggests that the gene expression of the basal group is most distinct from the TNF-induced no-treatment group and that the ISIS 25291 group can be separated from the induced and 8MM groups. Average linkage yielded a very similar result (dendrogram not shown).

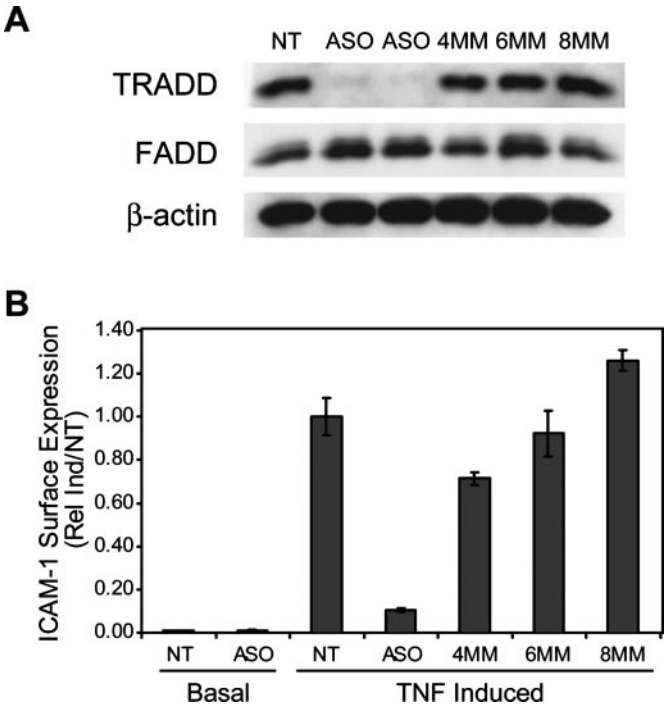

Fig. 1. The effects of ISIS 25291 on TNF induction of ICAM-1 in HUVECs is sequence-specific. HUVECs were treated daily for 2 days with either no oligonucleotide (NT), or with 75 nM of ISIS 25291 (ASO), ISIS 100720 (4MM), ISIS 110731 (6MM), or ISIS 110732 (8MM). A, on day 3, protein samples were harvested from one of four replicates for each treatment, and immunoblot analysis was performed on total protein to detect TRADD (top), FADD (middle), and β-actin (bottom) to verify TRADD knockdown, the absence of nonspecific activity, and equal protein loading, respectively. B, remaining cells were incubated in normal growth medium with or without TNF (5 ng/ml) for 18 h and harvested for determination of ICAM-1 protein surface expression by FACS analysis. Values represent the averages of three replicates and error bars represent S.D. of the replicates.

Genes in which mRNA expression levels were induced at least 2.5-fold by TNF, as assayed by Affymetrix U95Av2 high-density array chips, are shown in Table 3. The gene expression data were prefiltered on the present/absent calls determined by the Affymetrix Microarray Suite software. Probe sets were removed if none of the four groups [Bas, no-treatment (NT), 8MM, and ASO] showed as a present call. Twenty of the 24 genes (83%) that displayed an increase of >5-fold in TNF-induced expression levels demonstrated a significant reduction ($p \leq 0.05$) in expression levels relative to both the NT and 8MM groups as a consequence of treatment by ISIS 25291. Fewer genes were affected by ISIS 25291 treatment (56%) in the set that displayed an increase of <5-fold in TNF-induced expression levels relative to basal.

TNF-dependent increase in expression levels of hallmark pro-inflammatory genes (e.g., ICAM-1, VCAM-1, and IL-8) showed a significant reduction in HUVECS treated with ISIS 25291. E-selectin showed a moderate reduction (65% Ind) in

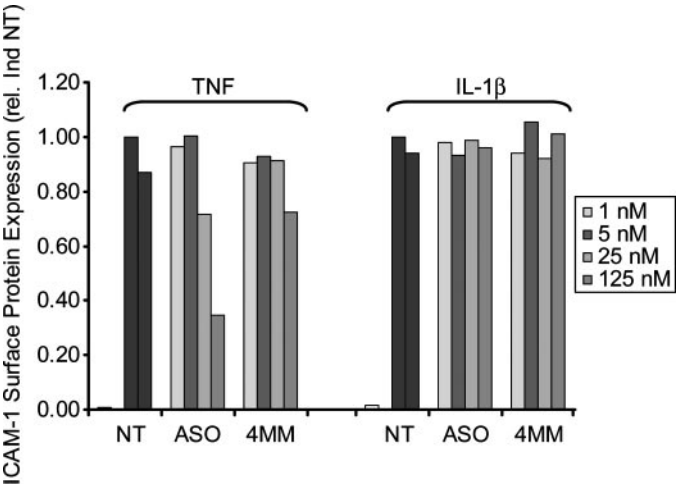

Fig. 2. Depletion of TRADD inhibits CAM induction by TNF but not IL-1β. HUVECs were treated with either no oligonucleotide (NT), or ISIS 25291 (ASO) and the 4-base mismatch control (4MM), ISIS 100720, at 1, 5, 25, or 125 nM. Forty-eight hours after treatment, cells were incubated in normal growth medium with or without TNF (5 ng/ml) or IL-1β (10 ng/ml) for 18 h, after which cell were harvested for FACS analysis of ICAM-1 surface expression levels.

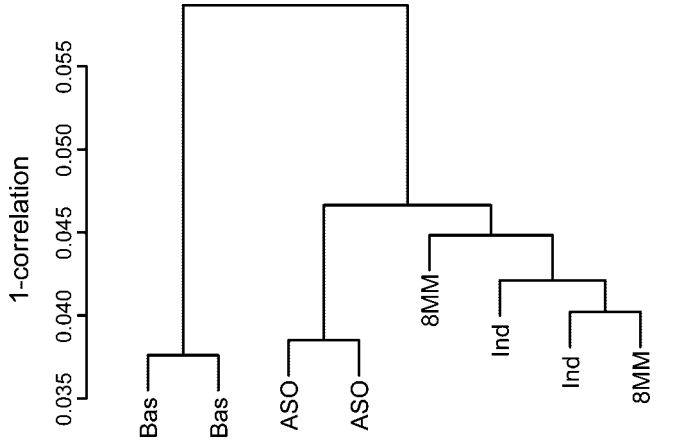

Fig. 3. Hierarchical clustering of Affymetrix U95A GeneChip microarray expression data. One minus the Pearson correlation coefficient was used as a dissimilarity measure, and complete linkage was used as a linkage method, where groups were untreated basal (Bas), untreated TNF-induced (Ind), and oligonucleotide-treated TNF-induced (8MM or ASO) as described in Table 3.

TABLE 3

Affymetrix U95A GeneChip analysis of RNA from TNF-induced HUVECs post-treatment with ISIS 25291

List of genes displaying a 2.5-fold, or greater, increase in expression in the TNF-induced no treatment group (NT) relative to the non-induced no treatment basal group (Bas). HUVECs were treated with 100 nM of either the eight mismatch control oligonucleotide, ISIS 110732 (8MM), or the anti-TRADD oligonucleotide, ISIS 25291 (ASO), for 4 h. Cells were then incubated in normal growth medium for an additional 68 h before induction with TNF (5 ng/ml) for 4 h.

RefSeq ID Number	TNF-Induced FC			%Ind-NT ASO	Gene Symbol	Gene Name
	NT	8MM	ASO			
NM_001078	601.9	703.8	200.5	33.3	VCAM1	Vascular cell adhesion molecule 1 (CD106)[a]
NM_000201	101.0	107.9	48.2	47.7	ICAM1	Intercellular adhesion molecule 1 (CD54)[a]
NM_005658	74.8	69.7	33.3	44.5	TRAF1	TNF receptor-associated factor 1[a]
NM_001781	50.4	37.8	36.6	72.6	CD69	CD69 antigen (p60, early T-cell activation antigen)
NM_014470	41.5	36.4	22.5	54.2	RND1	Rho family GTPase 1 (RHO6)[a]
NM_002090	34.7	36.9	16.5	47.6	CXCL3	Chemokine (C-X-C motif) ligand 3 (GRO3, MIP-2B)[a]
NM_001165	34.3	30.8	33.1	96.5	BIRC3	Baculoviral IAP repeat-containing 3 (cIAP2)
NM_006398	30.8	22.3	5.9	19.2	UBD	Diubiquitin D (FAT10)[a]
NM_006290	28.0	25.0	11.7	41.8	TNFAIP3	Tumor necrosis factor, α-induced protein 3[a]
NM_002089	23.9	26.2	13.5	56.5	CXCL2	Chemokine (C-X-C motif) ligand 2 (GRO2, MIP-2A)[a]
NM_000758	22.8	25.5	6.7	29.4	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)[a]
NM_002996	20.1	18.7	7.0	34.8	CX3CL1	Chemokine (C-X3-C motif) ligand 1 (fractalkine, neurotactin, SCYD1)[a]
NM_003855	14.8	17.2	8.4	56.8	IL18R1	Interleukin 18 receptor 1[a]
NM_000584	14.8	13.5	9.1	61.5	IL8	Interleukin 8[a]
NM_006291	13.6	17.2	6.6	48.5	TNFAIP2	Tumor necrosis factor, α-induced protein 2[a]
NM_003821	13.4	18.7	7.1	53.0	RIPK2	Receptor-interacting serine-threonine kinase 2[a]
NM_005409	13.3	10.7	5.5	41.4	CXCL11	Chemokine (C-X-C motif) ligand 11 (SCYB11)[a]
NM_020529	12.1	13.0	8.4	69.4	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α[a]
NM_001511	12.0	14.2	7.4	61.7	CXCL1	Chemokine (C-X-C motif) ligand 1 (MGSA, GRO1)[a]
NM_002982	11.1	10.7	5.5	49.5	CCL2	Chemokine (C-C motif) ligand 2 (MCP1, SCYA2)[a]
NM_001451	7.2	13.4	7.7	106.9	FOXF1	Forkhead box F1
NM_002198	6.8	9.6	3.3	48.5	IRF1	Interferon regulatory factor 1[a]
NM_003810	6.1	6.0	6.7	109.8	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10 (TRAIL)
NM_004049	5.5	6.3	3.5	63.6	BCL2A1	BCL2-related protein A1[a]
NM_004556	4.9	5.0	4.7	95.9	NFKBIE	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon
NM_020311	4.7	5.3	3.2	68.1	CMKOR1	Chemokine orphan receptor 1[a]
NM_004428	4.5	4.4	2.7	60.0	EFNA1	Ephrin-A1[a]
NM_005238	4.3	4.8	1.9	44.2	ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)[a]
NM_002999	4.1	7.0	2.7	65.9	SDC4	Syndecan 4 (amphiglycan, ryudocan)[a]
NM_002341	3.6	2.9	1.4	38.9	LTB	Lymphotoxin β (TNF superfamily, member 3)[a]
NM_021980	3.5	3.6	2.1	60.0	OPTN	Optineurin
NM_000395	3.4	3.9	2.5	73.5	CSF2RB	Colony stimulating factor 2 receptor, β, low-affinity (granulocyte-macrophage)
NM_005902	3.4	3.1	2.7	79.4	MADH3	MAD, mothers against decapentaplegic homolog 3 (Drosophila)
NM_003489	3.4	3.6	3.3	97.1	NRIP1	Nuclear receptor interacting protein 1
NM_014350	3.3	4.2	2.0	60.6	TNFAIP8	Tumor necrosis factor, α-induced protein 8 (GG2–1)[a]
NM_015589	3.3	3.2	3.2	97.0	SAMD4	Sterile α motif domain containing 4 (KIAA1053)
NM_001918	3.2	2.7	2.6	81.3	DBT	Dihydrolipoamide branched chain transacylase
NM_003709	3.2	2.8	3.0	93.8	KLF7	Kruppel-like factor 7 (ubiquitous)
NM_004209	3.1	3.1	2.9	93.5	SYNGR3	Synaptogyrin 3
NM_015022	3.0	2.5	1.4	46.7	PDZK3	PDZ domain containing 3[a]
NM_012294	3.0	3.4	2.3	76.7	GFR	Guanine nucleotide exchange factor for Rap1; M-Ras-regulated GEF[a]
NM_002192	2.8	3.3	3.0	107.1	INHBA	Inhibin, βA (activin A, activin AB α polypeptide)
NM_021127	2.8	3.7	3.5	125.0	PMAIP1	Phorbol 12-myristate 13-acetate-induced protein 1 (NOXA)
NM_003711	2.7	2.8	1.9	70.4	PPAP2A	Phosphatidic acid phosphatase type 2A[a]
NM_015033	2.6	2.4	1.4	53.8	FNBP1	Formin-binding protein 1[a]
NM_012420	2.5	3.7	1.5	60.0	IFIT5	Interferon-induced protein with tetratricopeptide repeats 5[a]
NM_001945	2.5	2.9	2.7	108.0	DTR	Diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)

* Genes that showed a significant reduction ($p \leq 0.05$) in mRNA expression levels in the ISIS 25291 treated cells relative to both untreated and mismatch control treated cells.

the microarray analysis but failed the *t* test selection criteria. However, analysis by real-time quantitative RT-PCR showed a significant reduction of E-selectin mRNA levels in the ISIS 25291-treated samples (41% Ind, $p \leq 0.05$). Furthermore, ASO-specific reductions in mRNA expression levels were observed for a number of functionally distinct TNF-induced genes, including chemokines (CXCL1, CXCL2, CXCL3, CXCL11, and CX3CL1), cytokines (CSF2 and LTB), receptors (IL18R and CMKOR1), antigen-processing components (UBD), kinases (RIPK2), TNF receptor-associated factors (TRAF1), regulators of apoptosis (BCL2A1, TNFAIP3, and TNFAIP8), inhibitors of NF-κB signaling (TNFAIP3 and NFKBIA), and transcription factors (IRF1 and ETS1). Expression level profiles of TRADD, ICAM-1, VCAM-1, TNFAIP3 (A20), IRF-1, and CSF2 (GM-CSF) were confirmed by quantitative RT-PCR analysis (data not shown). TNF-induced genes that were not affected by TRADD knockdown included CD69, BIRC3 (cIAP2), TNFSF10 (TRAIL), and FOXF1.

Depletion of TRADD Inhibits TNF-Mediated Activation Events of the NF-κB and JNK Pathways. The effect of ISIS 25291 treatment on TNF mediated degradation of IκB-α, and phosphorylation of c-jun was undertaken to confirm the mechanism by which TRADD depletion elicits its repressive effects on TNF-mediated induction of proinflammatory genes. Treatment of HUVECS with TNF resulted in the activation of both the NF-κB and JNK signaling pathways, as reflected by the degradation of IκB-α and the phosphorylation of c-jun, respectively (Fig. 4). TRADD depletion by ISIS 25291 in HUVECs resulted in partial inhibition of IκB-α degradation in cells stimulated with TNF but not with IL-1β (Fig. 4). TRADD depletion also resulted in decreased phosphorylation of c-jun after stimulation with TNF but not IL-1β. In both cases, the mismatch control had no significant inhibitory effect, indicating that the effects observed in cells treated with ISIS 25291 were sequence specific.

Discussion

ISIS 25291 is an antisense oligonucleotide that reduces TRADD mRNA levels in a sequence-dependent manner to result in depletion of TRADD protein levels. Depletion of TRADD by ISIS 25291 reduced TNF-induced activation of the NF-κB and JNK pathways, as well as RNA expression levels of a number of TNF inducible genes. These results indicate that TRADD is required for full activation of TNF signal transduction pathways, which lead to increased gene expression levels.

Although TNF and IL-1β promote similar phosphorylation events and induce expression of a significant number of the same genes (Zhao et al., 2003), they do so through usage of different receptor adaptor proteins (O'Neill and Greene, 1998; Wajant et al., 2001; MacEwan, 2002). Identification of these adaptor proteins has frequently been achieved through usage of the yeast two-hybrid system, using the receptors as bait. In this manner, TRADD was identified to interact directly with TNF-R1. Further analysis with a TRADD cDNA expression construct demonstrated that TRADD did not directly interact with structurally or functionally related receptors (Hsu et al., 1995) [e.g., tumor necrosis factor receptor 2 (TNF-R2), Fas antigen (Fas), or interleukin 1 receptor, type I]. In support of these results, we have found that ASO knockdown of TRADD only affects TNF signaling, with no observable affect on IL-1β signaling.

In contrast to the selective role of TRADD in TNF signaling, c-raf kinase and Ha-ras are two intermediate signaling components that have been found to be involved in induction of CAM expression in human endothelial cells by both TNF and IL-1β (Xu et al., 1998). In the case of TNF, the predominant effect of knockdown of either c-raf kinase or Ha-ras was reduction of E-selectin expression, followed by VCAM-1 and a limited reduction of ICAM-1. Furthermore, it was found that TNF activation of the JNK and extracellular signal-regulated kinase pathways is dependent upon c-raf kinase, and induction of E-selectin is solely dependent upon activation of the JNK2 isoform. Knockdown of TRADD, on the other hand, reduced expression of all three CAMs, with a more profound effect on VCAM-1 and ICAM-1 expression. The modest level of phosphorylated c-jun observed in cells treated with ISIS 29591 may be, in part, the basis of the differences in CAM expression profiles.

Gene expression levels that were unaffected by TRADD knockdown in HUVECs may reflect signaling through other TNF-R1 adaptor proteins [e.g., FAN, Grb2, or MADD (MacEwan, 2002; Madge and Pober, 2001)]; alternatively, TNF signaling through the second TNF receptor, TNF-R2 (Slowik et al., 1993; Paleolog et al., 1994; Cheng and Chen, 2001). With respect to TNF-R2, TNF-induced expression of cellular adhesion molecules (ICAM-1, VCAM-1, and E-selectin), IL-8,

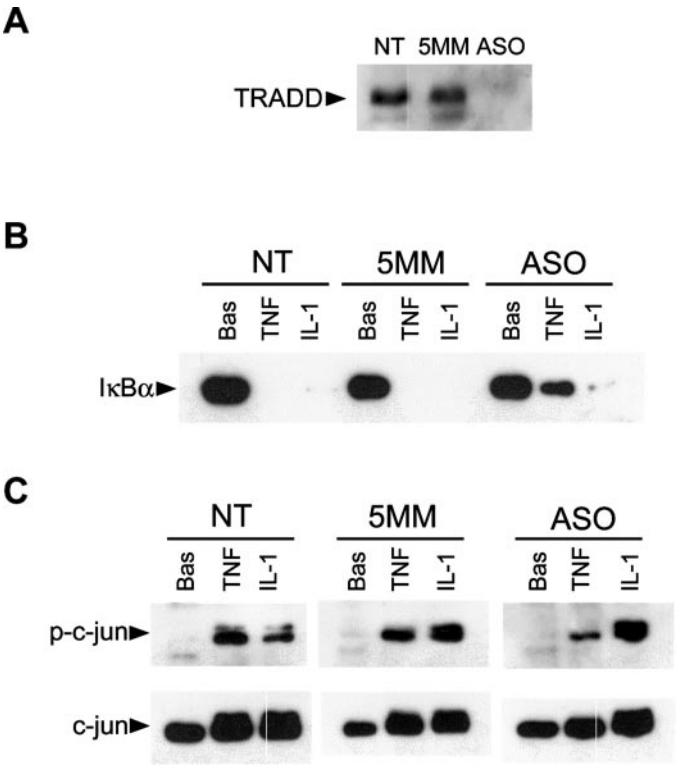

Fig. 4. IκB-α degradation and c-jun phosphorylation proceed via a TRADD-dependent pathway. HUVECs were treated with no oligonucleotide (NT) or 50 nM concentrations of either the mismatch control ISIS 25290 (5MM) or ISIS 25291 (ASO). Cells were reincubated for 48 h before a second identical treatment. Cells were then reincubated for 72 h. Cells receiving treatment above were left uninduced (Bas) or induced with TNF (10 ng/ml) or IL-1β (10 ng/ml) for 30 min. Cell lysates were subjected to Western blot analysis for protein expression levels of TRADD (A), IκB-α (B), and c-jun and phospho c-jun (C).

GM-CSF, and tissue factor was reduced in HUVECs treated with antagonistic antibodies to either receptor, although to a lesser degree by the TNF-R2 antibody (Paleolog et al., 1994). Furthermore, a more recent report has shown that neutralizing antibodies to either receptor, individually, partially inhibits TNF-induced expression of Ephrin A1 mRNA in HUVECs (Cheng and Chen, 2001). These antibody-mediated blocking results, however, may reveal a role of TNF-R2 in "ligand-passing" to TNF-R1 rather than in direct signaling (MacEwan, 2002).

The mechanism responsible for the attenuation of TNF-mediated induction of the various proinflammatory molecules by TRADD knockdown involves partial blockade of signal transduction pathways that regulate the transcription factors that activate their expression. NF-κB and AP-1 are key transcription factors involved in TNF-induced activation of gene expression (Baud and Karin, 2001). In this respect, a substantial number of the TNF-induced genes identified in the DNA microarray analysis harbor NF-κB binding sites in their promoter regions (Pahl, 1999; Schmid and Adler, 2000); including VCAM-1, ICAM-1, TRAF1, CD69, CXCL3, BIRC3, TNFAIP3, CXCL2, CSF2, IL8, CXCL11, NFKBIA, CXCL1, IRF1, and BCL2A1. Regulation of NF-κB activity occurs via several mechanisms, such as post-translational processing, phosphorylation, and interaction with the NF-κB inhibitor proteins. In this study, we further examined the effects of TRADD knockdown on NF-κB activity by analysis of IκB-α, the most well characterized member of IκB family of inhibitor proteins. IκB-α inhibits NF-κB activity through interactions that mask the transcription factor's nuclear localization signal to result in its sequestration in the cytoplasm. Degradation of IκB-α occurs upon TNF induction to result in release and translocation of NF-κB to the nucleus, where it activates transcription of its target genes. The effects of inhibition of TNF-induced degradation of IκB-α by TRADD knockdown is reflected by the reduced expression levels of the majority of the genes whose expression is regulated by NF-κB activity.

A fewer number of TNF-induced genes were identified in the DNA microarray data, which have been indicated to contain AP-1 binding sites in their promoter region. These included VCAM-1, E-selectin, CSF2 (GM-CSF), IL8, CD69, BIRC3 (cIAP2), and TNFSF10 (TRAIL). AP-1 binding complexes are composed of homo- or heterodimers that are derived from the Jun, Fos, ATF/CREB, and Maf transcription factor subfamilies (Shaulian and Karin, 2001; Dunn et al., 2002). TNF-induction of AP-1 activity is predominantly mediated by the JNK and p38 MAPK pathways, for which c-jun (JNK only) and ATF-2 (JNK and p38) are known substrates. In this study, we further examined the effects of TRADD knockdown on AP-1 activity by evaluation of the level of phosphorylated c-jun. The degree of TNF-induced phosphorylation of this transcription factor displayed a direct correlation with TRADD protein levels, indicative of a corresponding loss of AP-1 transcription activity upon TRADD depletion. It is interesting that several of the genes whose expression was induced independently of TRADD protein levels[e.g., CD69 (Lopez-Cabrera et al., 1995) and TRAIL (Wang et al., 2000)] have been shown to contain AP-1 binding sites in their promoter regions. TRADD-independent expression of such molecules as CD69 and TRAIL may reflect a limited understanding of the promoter regions of the respective genes and/or the interplay between putative transcription regulators within the AP-1 family and other families.

Other receptors that directly interact with TRADD include DR3 (Chinnaiyan et al., 1996), DR6 (Pan et al., 1998), $p75^{NTR}$ (Cantarella et al., 2002; El Yazidi-Belkoura et al., 2003), and LMP1 (Eliopoulos et al., 1999; Izumi et al., 1999). All the receptors, except for LMP1, interact with TRADD via their complementary death domain regions. LMP1 is unique in that it does not contain a death domain; as such interacts with the N terminal region of TRADD via its C terminal domain. TRADD's role in signaling from each of these receptors is similar in that it recruits or enhances association and activation of other components (e.g., TRAF2 and RIP) to affect signal transmission. The finding that ISIS 25291 promotes sufficient depletion of TRADD to block TNF induction of various pro-inflammatory genes indicates TRADD as a potential cellular target for modulating diseases associated with TNF activity. Extrapolation to other pathological processes (e.g., tumor angiogenesis) that engage other receptors that interact with TRADD may also prove to be of value.

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