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Cutting Edge: TNF-Induced MicroRNAs Regulate TNF-Induced Expression of E-Selectin and Intercellular Adhesion Molecule-1 on Human Endothelial Cells: Feedback Control of Inflammation

Yajaira Suárez,1 Chen Wang, Thomas D. Manes, and Jordan S. Pober

MicroRNAs (miRNAs) pair with target sequences in the 3′ untranslated region of mRNAs to posttranscriptionally repress gene expression. In this study, we report that TNF-mediated induction of endothelial adhesion molecules can be regulated by miRNAs that are induced by TNF. Specifically, E-selectin and ICAM-1 are targets of TNF-induced miRNAs miR-31 and miR-17-3p, respectively. Specific antagonism of these TNF-induced miRNAs increased neutrophil adhesion to cultured endothelial cells. Conversely, transfections with mimics of these miRNAs decreased neutrophil adhesion to endothelial cells. These data suggest that miRNAs provide negative feedback control of inflammation. The Journal of Immunology, 2010, 184: 21–25.

The proinflammatory cytokine TNF induces de novo expression of E-selectin (SELE), ICAM-1, and VCAM-1, endothelial cell (EC) surface proteins that bind leukocytes (1–3). MicroRNAs (miRNAs) control gene expression by pairing with partially complementary target sites in mRNA 3′ untranslated regions (UTRs), resulting in translational repression and/or mRNA destabilization (4–6). Most miRNAs are constitutively expressed in a lineage-specific manner that peaks during embryological development, but some miRNAs are regulated dynamically in the adult organism. Vascular endothelial growth factor (VEGF) induces a subset of EC miRNAs that regulate cell growth, survival, and angiogenesis (7–9). Other miRNAs are induced in different cell types by a variety of proinflammatory stimuli (10–15). A constitutively expressed, EC-restricted miRNA (miR-126) modulates TNF-induced VCAM-1 expression (16). In this study, we report that TNF increases miR-155, -31, -17, and -191 without changing miR-20a, -222, and -126 in human ECs and that SELE and ICAM-1 are targets of TNF-induced miR-31 and -17-3p, respectively, regulating neutrophil binding to ECs. These data reveal how inducible miRNAs make up a negative feedback loop to control inflammation.

Materials and Methods

Cell culture

Human cells were obtained from discarded tissues or peripheral blood of deidentified donors under protocols approved by the Yale Human Investigation Committee. HUVECs and human dermal fibroblasts (HDFs) were isolated and cultured as described previously (8, 17, 18). Neutrophils were isolated from whole blood of healthy adult donors (19).

miRNA array analyses

HUVEC miRNA expression was assessed following stimulation for 2 or 24 h with 10 ng/ml TNF or buffer control using Exiqon miRCURY LNA Arrays as described (8). The data have been deposited in MIAMExpress with ArrayExpress accession number E-MTAB-150 and are accessible through www.ebi.ac.uk/microarray-as/ae/. Array results were validated by Northern blot (8, 18) or by quantitative RT-PCR using a mirVanaTM qRT-PCR miRNA Detection Kit (Ambion, Austin, TX) (8, 18).

Reporter gene assays

cDNAs encoding the entire 3′ UTR of ICAM-1 (1,329 kb) or SELE (1,883 kb) mRNAs were amplified by RT-PCR from total HUVEC RNA using Xhol and NotI linker/primers and directionally cloned downstream of the Renilla luciferase open reading frame in the psiCHECK2 vector (Promega, Madison, WI) that also contains a constitutively expressed firefly luciferase gene. ICAM-1 or SELE 3′ UTRs were cloned in reverse orientation as controls lacking the miRNA target sequence (20). Additionally, the region complementary to the miR-31 seed sequence in position 94–100 of the human SELE 3′ UTR, TCTTGCC, was scrambled to CTGCCTT (mSELE 3′ UTR), and the region complementary to the miR-17-3p seed sequence in positions 638–644 and 1148–1154 in human ICAM-1 3′ UTR, ACTGCAG, was scrambled to GTAAGCC (mICAM-1 3′ UTR). All constructs were confirmed by sequencing, COS-7 cells (gift of Dr. William Sessa, Yale University) were cotransfected with 1 μg the indicated reporter construct and the indicated miRIDIAN miRNA mimic or negative control mimic sequences (CM) (Dharmacon, Lafayette, CO) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Renilla luciferase activity was normalized to the corresponding firefly luciferase activity.

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Abbreviations used in this paper: CI, control inhibitor sequence; CM, control mimic sequence; EC, endothelial cell; HDF, human dermal fibroblast; miRNA, microRNA; QT/PCR, quantitative real-time PCR; SELE, E-selectin; UTR, untranslated region; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor.

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using a Dual-Glo Luciferase Assay System (Promega) and reported as the percentage of control cells cotransfected with the same concentration of CM.

**HUVeC transfection**

HUVeCs were transfected with 40 nM miRIDIAN miRNA mimics or with 60 nM miRIDIAN miRNA inhibitors (Dharmacon) using Oligofectamine (Invitrogen) (8, 18). Control samples were transfected with an equal concentration of CM or negative control inhibitor sequence (CI), as described previously (8). The effects of transfections with miR-mimics/inhibitors was assessed by quantitative real-time PCR (QRT-PCR). miR-31 and -17-3p levels were efficiently increased after 18 h and persisted 36 h following transfection, and transfection with miR-31 did not affect the endogenous levels of miR-17-3p and vice versa (not shown). Transfection with miRNA inhibitors for miR-31 or -17-3p efficiently reduced the TNF-stimulated levels of miR-31 and -17-3p below levels in untreated cells, indicating an efficient inhibition of indicated miRNAs (not shown).

**Western blotting**

Western blots were performed (8, 18) using mouse mAbs reactive with human ICAM-1 and SELE (R&D Systems, Minneapolis, MN) under nonreducing conditions or with Tie2 and vascular endothelial cadherin (VE-cadherin) polyclonal Abs (Santa Cruz Biotechnology, Santa Cruz, CA) or Hsp-90 mAb (BD Biosciences, San Jose, CA) under reducing conditions. Binding of fluorophore-conjugated secondary Abs (Rockland, Gilbertsville, PA) were visualized using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

**Cell surface immunostaining**

HUVeCs were transfected and/or treated with TNF as indicated, stained with directly fluor-conjugated mAbs as described (18), and analyzed on a FACS-calibur (BD Biosciences) using CellQuest analysis software, collecting 5000 gated cells per sample. Specific Abs used in these analyses were FITC or PE directly conjugated and reactive with human SELE (R&D Systems) or ICAM-1 (BD Pharmingen, San Diego, CA), respectively.

**Neutrophil adhesion assays**

Adhesion of neutrophils to the confluent EC monolayer was measured as described previously (19) with slight modifications. Neutrophils were labeled with calcein-AM (Molecular Probes, Eugene, OR) for 10 min at 37°C and suspended in PBS containing 1 mM magnesium chloride, 0.5 mM calcium chloride, and 0.1 g/l glucose (PBS plus). Transfected HUVeCs were treated with 10 ng/ml TNF for 6 h, washed twice in PBS plus, and then incubated with 1 ml labeled neutrophils (10⁶) for 15 min at 37°C under static conditions. Samples were fixed, and bound neutrophils were counted in five randomly selected ×10 fields. Where indicated, HUVeCs were incubated with anti-SELE and/or anti-ICAM-1 blocking Abs (R&D Systems) at 50 or 10 µg/ml, respectively, for 1 h before the addition of the neutrophils.

**Statistical analyses**

Statistical differences between groups were assessed by the two-tailed paired Student t test. Data from QRT-PCR and reporter assays were expressed relative to the control of each experiment, and 95% confidence intervals were calculated; differences were judged significant when the confidence interval did not include the “1” or “100” value.

**Results and Discussion**

We characterized miRNA expression in HUVeCs stimulated for 2 or 24 h with 10 ng/ml TNF using microarrays. Several miRNAs were induced after 2 h; some of these had increased after 24 h, whereas others had decreased (Fig. 1, Supplemental Table I). Levels of selected miRNA species identified in the arrays (Fig. 1, highlighted in black) were confirmed by Northern blotting (Supplemental Fig. 1A) and/or by QRT-PCR (Supplemental Fig. 1B). Levels of miR-155, -31, -17-5p, -191, and -126b were increased by TNF, but levels of miR-222, -20a, and -126 were not (Supplemental Fig. 1A, 1B).

Several TNF-induced HUVEC miRNAs were not induced in HDFs, suggesting at least partial specificity of the EC response. miR-155 is highly induced by TNF in HUVeCs and HDFs (Supplemental Fig. 1C). TNF also induces miR-155 in immune cells (12), in synovial fibroblasts (21), and in lymphomas (9, 22). miR-155 is also upregulated in ECs by VEGF (8).

Other miRNAs induced by TNF also overlap with those induced by VEGF (Supplemental Fig. 2). VEGF induces several components of the c-myc/E2F–regulated oncogenic cluster miR-17/92 (including miR-17-5p, -18a, and -20a) (8), but only miR-17-5p is upregulated by TNF (Fig. 1, Supplemental Fig. 1A). Although a single primary RNA transcript encodes the entire cluster, differences in the efficiency of posttranscriptional processing (23, 24) or selective blockade of pri-miRNA processing (25) may result in differential expression of mature miRNAs. miR-17-5p is upregulated in psoriasis, atopic eczema, and systemic lupus erythematosus (26, 27). In human pulmonary aortic ECs, the miR-17-92 cluster is modulated by IL-6 via STAT3 (28). Secretion of IL-6 is induced in ECs by TNF (29), raising the possibility of an indirect autocrine/paracrine effect. In HUVeCs, guide and passenger strands of miR-17 (5p and 3p, respectively) are induced by TNF (Supplemental Fig. 1A).

We used miRNA target prediction algorithms (5) to identify a putative binding site in the 3′ UTR of SELE for miR-31 (Supplemental Fig. 3A), a highly TNF-induced miRNA (Fig. 1), and an additional site for miR-221 and -222 (Supplemental Fig. 3A), two highly homologous miRNAs, derived from the same pri-miRNA transcript that contains identical seed sequences and that are not regulated by TNF (Fig. 1). The miR-31 predicted site is a canonical 7mer-m8 site supported by an additional 3′ pairing optimally centered on miRNA nt 13–16 (5). The human “seed” region of miR-31 is critical for the sequence complementary to the miR-31 seed sequence (mSELE 3′ UTR) (Fig. 2A). Luciferase activity of SELE 3′ UTR was significantly reduced with as little as 10 nM of miR-31 (Supplemental Fig. 4A). Luciferase activity of SELE...
miR-31 inhibitor (I-miR-31) prior to TNF stimulation (Fig. 6 h, respectively, when cells were transfected with an antisense
Furthermore, SELE levels increased by 25% and 20% at 3 and
reduced TNF-induced SELE levels by
expression, respectively (1, 17). By immunoblotting, M-miR-31
TNF for 3 or 6 h, the peaks of SELE mRNA and protein ex-
for 12 h; after a 24-h recovery period, they were stimulated with
HUVECs were transfected with miR-31 mimic (M-miR-31)
9
B
duced miR-17-3p, -17-5p, -155, or -125b (Fig. 2
3
efficacy of 6mer sites (5) may explain this lack of effect. SELE
3
sites (5) in the human 3
9
UTR luciferase activity also was not affected by TNF-in-
duced miR-17-3p, -17-5p, -155, or -125b (Fig. 2B), none of
which is predicted to bind to the SELE 3’UTR.
To analyze the effect of miR-31 on SELE protein expression,
HUVECs were transfected with miR-31 mimic (M-miR-31)
for 12 h; after a 24-h recovery period, they were stimulated with
TNF for 3 or 6 h, the peaks of SELE mRNA and protein ex-
pression, respectively (1, 17). By immunoblotting, M-miR-31
reduced TNF-induced SELE levels by ≈35% at 3 h (Fig. 2C).
Furthermore, SELE levels increased by 25% and 20% at 3 and
6 h, respectively, when cells were transfected with an antisense
miR-31 inhibitor (I-miR-31) prior to TNF stimulation (Fig.
2D). Two other EC proteins, VE-cadherin and Tie2, were not
changed by these treatments (although a putative binding site
for miR-31 was predicted in Tie2). By FACS, TNF-induced
SELE cell surface expression increased when miR-31 was in-
hibited (Supplemental Fig. 5A).

Two canonical 7mer-m8 and one 7mer-A1 putative binding
sites (5) in the human 3’UTR of ICAM-1 were predicted for the
TNF-induced miR-17-3p and for non-TNF-induced miR-
221/222, respectively (Supplemental Fig. 3B). None of these
sites is conserved across species but were independently pre-
dicted by three different target-prediction algorithms (Tar-
getScan, miRBase, and RegRNA), and a large fraction of
nonconserved sites can be functional (30). In reporter gene
assays, miR-17-3p efficiently repressed ICAM-1 3’UTR luci-erase activity by ≈40% (Fig. 3A). Luciferase activity was un-
affected by cotransfection of CM, and miR-17-3p did not affect
reporter vectors lacking ICAM-1 3’UTR, with reverse-ori-
ented 3’UTR (control 3’UTR) or with a mutation in the se-
quence complementary to the miR-17-3p seed sequence
(miCAM-1 3’UTR). Luciferase activity of ICAM-1 3’UTR
was significantly reduced at 40 nM (Supplemental Fig. 4B).
ICAM-1 3’UTR luciferase activity was unaffected by miR-221
or -222 (Fig. 3B), suggesting the predicted site is nonfunctional
(5). It was recently reported that miR-222 can regulate the
expression of ICAM-1 in tumor cells by direct interaction
with its 3’UTR (31). A key difference from our experiments
is that Ueda et al. (31) tested the isolated target sequence rather
than testing the sequence in the context of the entire 3’UTR.
Contextual features of the 3’UTR, such as secondary struc-
tures or local AU-rich regions, among others, can govern

![FIGURE 2](image_url)

**FIGURE 2.** miR-31 regulates TNF-induced SELE expression by targeting the SELE 3’UTR. COS-7 cells were cotransfected with the indicated constructs and with 40 nM of M-miR-31 or CM (A) or with SELE 3’UTR construct and 40 nM of the indicated miRNA mimics or CM (B). Data are expressed as relative luciferase activity to control samples cotransfected with an equal concentration of CM (mean ± SEM of three experiments performed in duplicate). *Significantly different from TNF-treated control, p ≤ 0.05.

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![FIGURE 3](image_url)

**FIGURE 3.** miR-17-3p regulates TNF-induced ICAM-1 expression by targeting the ICAM-1 3’UTR. COS-7 cells were cotransfected with the indicated constructs and with 40 nM of M-miR-17-3p or CM (A) or with ICAM-1 3’UTR construct and 40 nM of the indicated miRNA mimics or CM (B). Data are expressed as relative luciferase activity to control samples cotransfected with an equal concentration of CM (mean ± SEM of three experiments performed in duplicate). *Significantly different from TNF-treated control, p ≤ 0.05.
miRNA–mRNA interactions (5). ICAM-1 3′ UTR luciferase activity was not affected by other TNF-induced miRNAs (miR-31, -17-3p, -155, and -125b) (Fig. 3B) not predicted to bind to the ICAM1 3′ UTR. In general, the miRNA strand that has the lowest thermodynamic stability at its 5′-terminus acts as the mature miRNA (guide strand), and the other (passenger) strand (miRNA*) is degraded. However, in some cases, both miRNA strands may accumulate in tissues at significant levels (32), and there are validated examples of trans-regulatory RNAs with demonstrable activities (33). Although basal miR-17-3p levels are very low compared with miR-17-5p levels, miR-17-3p is clearly stimulated upon TNF treatment (Supplemental Fig. 6).

To analyze the effect of miR-17-3p on ICAM-1 protein expression, HUVECs were transfected with miR-17-3p mimic (M-miR-17-3p) for 12 h and, after a 24-h recovery period, they were stimulated with TNF for 12 h. M-miR-17-3p reduced TNF-induced ICAM-1 expression by ≈30% (Fig. 3C). ICAM-1 levels increased up to 35% when cells were transfected with an antisense miR-17-3p inhibitor (I-miR-17-3p) prior to TNF stimulation (Fig. 3D). Tie2 levels were not affected by M-miR-17-3p (Fig. 3C, 3D). Although the total protein levels were consistently induced by miR-17-3p antagonism, increases in TNF-induced ICAM-1 cell surface expression observed by FACS did not reach statistical significance (Supplemental Fig. 5B). The increase was significant in some HUVEC donors, consistent with a polymorphic response.

We did not find predicted sites for regulation of VCAM-1 by TNF-induced miRNAs. miR-126 is an EC-specific miRNA that was shown to regulate the expression of VCAM-1 and limit the adherence of leukocytes to EC (16); miR-126 is not regulated by TNF (Fig. 1) (16). miRGen Target interface does not predict any sites for miR-126 in the 3′ UTRs of SELE or ICAM-1.

Finally, we tested whether the miRNA effects on TNF-induced adhesion molecule expression are functional. As expected, TNF increased neutrophil adhesion to ECs. Exogenous overexpression of M-miR-31, M-miR-17-3p, or the combination of the two significantly reduced neutrophil binding (Fig. 4A, Supplemental Fig. 7A), whereas the inhibition of miRNAs miR-31 and/or M-miR-17-3p increased neutrophil adherence to TNF-stimulated ECs (Fig. 4B, Supplemental Fig. 7B). It is likely that TNF-induced miRNAs (miR-31 and -17-3p) regulate neutrophil adhesion through the regulation of TNF-induced expression of SELE and ICAM-1, respectively, although other actions of these miRNAs could affect neutrophil binding. To test this possibility, we performed experiments combining blocking Abs with miRNA mimic or inhibitor transfections. As expected, the incubation with blocking Abs alone profoundly reduced neutrophil binding (Supplemental Fig. 7C, 7D). Transfection of mimics did not cause any evidence of further reduction in TNF-induced neutrophil binding (Supplemental Fig. 7C). Transfection of inhibitors produced a small increase in neutrophil binding in the presence of blocking Abs that did not reach statistical significance (Supplemental Fig. 7D). These experiments do not rule out the possibility that these TNF-induced miRNAs affect neutrophil adhesion by actions on targets other than SELE or ICAM-1, but if such effects exist, they are too small to detect when interactions with SELE or ICAM-1 are blocked.

In summary, we illustrated two examples of miRNA-mediated feedback control of the expression of adhesion molecules (SELE and ICAM-1). This kind of regulation, in which miRNA-

FIGURE 4. Regulation of neutrophil adhesion by miR-31 and/or miR-17-3p. ECs transfected for 12 h with M-miR-31 and/or M-miR-17-3p or CM (B) or with I-miR-31 and/or I-miR-17-3p or CI (B). 24 h after the transfection period, the cells were treated or not with TNF. Calcein-AM–labeled neutrophils were added to the ECs and incubated at 37°C for 15 min. Graphs show the mean number of bound cells from triplicates ± SD of one experiment of three with similar results. *Significantly different from TNF-treated control, p ≤ 0.05.

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Disclosures
The authors have no financial conflicts of interest.

References


