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Verotoxin-1 stimulation of macrophage-like THP-1 cells up-regulates tissue factor expression through activation of c-Yes tyrosine kinase: Possible signal transduction in tissue factor up-regulation

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Abstract

Verotoxin (VT)-producing *Escherichia coli* (*E. coli*) O157:H7 infections are frequently complicated by thrombotic angiopathy, hemolytic uremic syndrome (HUS) and neurological symptoms. The present data demonstrate that VT-1 (Shiga toxin) stimulation of macrophage-like THP-1 cells up-regulates the activity, antigen and mRNA levels of tissue factor (TF), a key cofactor of the coagulation–inflammation–thrombosis circuit. This up-regulation is accompanied by phosphorylation of phosphatidylinositol 3-kinase (PI3-kinase), IκB kinase β (IKKβ) and extracellular signal-regulated kinase 2 (ERK2). Changes in TF mRNA levels were in parallel with the activation of NF-κB/Rel and Egr-1 activation, but not with AP-1. Inhibition of PI3-kinase attenuated VT-1-induced phosphorylation of IKKβ and ERK2, and the up-regulation of TF mRNA levels. VT-1 stimulation rapidly activated c-Yes tyrosine kinase, a member of the Src family. Treatment of the cells with c-Yes antisense oligos attenuated the VT-1-induced phosphorylation of PI3-kinase, IKKβ and ERK2, activations of NF-κB/Rel and Egr-1, and up-regulation of TF mRNA levels. These results suggest that VT-1-induced macrophage stimulation activates c-Yes, which then up-regulates TF expression through activation of the IKKβ/proteasome/NF-κB/Rel and MEK/ERK2/Egr-1 pathways via activation of PI3-kinase. Induction of macrophage TF expression by VT-1 may play an important role in the acceleration of the coagulation–inflammation–thrombosis circuit during infections by VT-producing *E. coli*.

Keywords: Tissue factor; Verotoxin-1; Shiga toxin; Blood coagulation; c-Yes; NF-κB; Macrophage-like cell

1. Introduction

Verotoxin (VT)-producing *Escherichia coli* O157:H7 (*E. coli* O157) is responsible for hemorrhagic colitis and the subsequent development of life-threatening complications, including acute renal failure and neurological symptoms [1]. The acute renal failure caused by VTs is referred to as hemolytic uremic syndrome (HUS) and is characterized by a glomerular thrombotic microangiopathy with fibrin-rich thrombi [2]. This could be caused by activation of coagulation and suppression of fibrinolysis, since circulating levels of the thrombin–antithrombin complex, prothrombin fragment 1+2, and plasminogen activator inhibitor-1 (PAI-1) are elevated in these patients [3]. VTs are thought to be essential for the development of HUS and neurological complications, since many of the histopathological features of the renal and central nervous system (CNS) vascular damage seen in humans can be reproduced after the administration of purified VTs in animals [4].

VT-1 is one of several verotoxins that include VT-1, VT-2 and variants of VT-2, and is identical to Shiga toxin produced by *Shigella dysenteriae*. The VT-1 holotoxin binds with high affinity to the glycolipid, globotriaosyl ceramide (Gb3), which is a cellular receptor for VT-1 on various target cells, and induces pathophysiological responses when activated [5]. Gb3 expression has been reported for various cells including renal tubular cells, vascular endothelial cells [6], gut epithelial cells [7], nervous system capillary cells [8], and monocyte/macrophage

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lineage [9]. Depletion of macrophages from mice in the VT-induced HUS experimental model resulted in reduction of the inflammatory cytokine production and the lethality of the model [10,11], which indicates that macrophages found within the kidney and CNS play a significant role in the pathogenesis of HUS and CNS. With regard to subcellular signaling, it has reported that production of the inflammatory cytokines including tumor necrosis factor (TNF)-α in VT-1-stimulated macrophages are correlated with activations of both p38 mitogen-activated protein kinase (p38 MAPK) and extracellular-regulated kinase (ERK) [12]. On the other hand, Katagiri et al. [13] reported that c-Yes, a member of the Src family kinases, is associated with Gb3 after VT-1 stimulation of renal tubular cells. However, the role of c-Yes in the expression control of proteins and the subcellular signaling in VT-1-stimulated cells has yet to be investigated.

Tissue factor (TF) is an integral member of the cytokine receptor family and triggers blood coagulation by binding with the enzyme coagulation factor VIIa and the substrate factor X [14]. In this ternary complex, Xa is generated and the liberated Xa leads to thrombin formation and fibrin deposition. Furthermore, signals from the TF–VIIa complex enhance cell migration via the protease-activated receptor 2 (PAR2) [15] and signals from the TF–VIIa–Xa ternary complex and/or Xa signals produce inflammatory cytokines via PAR1 and 2 [16]. Under normal circumstances, TF is either not expressed or only expressed at very low levels on the surface of endothelial cells and monocytes/macrophages [17]. However, after stimulation with bacterial lipopolysaccharide (LPS) and inflammatory cytokines, there is a marked expression of TF on the cell surfaces [18–20]. Aberrant TF expression is found in various conditions such as inflammation, septic shock, thromboembolic disorders, and disseminated intravascular coagulation (DIC) [21,22]. In the *E. coli*-induced sepsis model, TF antibody treatment or the use of mice that lack expression of the TF cytoplasmic domain leads to attenuation of renal failure abnormalities, tissue neutrophil influx, fibrin deposition, and increases in pro-inflammatory cytokines, such as IL-6 and 8 [23,24]. This implies that TF participates in the regulation of both the coagulation and inflammatory processes and plays a key role in the coagulation–inflammation–thrombosis circuit, which is responsible for the subsequent development of life-threatening complications [25]. However, there is no information about TF expression in monocyte/macrophage lineage cells stimulated with VT-1.

Monocyte/macrophage lineage cells exhibit a different response to VT-1 during the differentiation of these cells. Differentiation of the THP-1 cells, a human monocytic leukemia cell line, is initiated by treatment with phorbol 12-myristate 13-acetate (PMA) and mimics plastic-adherent macrophages (macrophage-like THP-1 cells) [26]. VT treatments of non-adherent monocytes and undifferentiated THP-1 cells result in rapid cell death. However, while adherent monocytes and differentiated macrophage-like THP-1 cells, as well as macrophages, are resistant to VT-1, they are responsible for VT-1 stimulation that leads to a subsequent cytokine production [27]. The differentiated macrophage-like THP-1 cells are widely used as macrophage substitutes in experiments designed to explore the effect of VT-1 on macrophages. The present study was undertaken to evaluate whether VT-1 induces TF expression on differentiated macrophage-like THP-1 cells through c-Yes activation and to elucidate the molecular basis for the transcriptional regulation of the TF gene after VT-1 induced c-Yes activation.

2. Materials and methods

2.1. Materials

Purified VT-1 was donated by Denka Seiken Co. (Niigata, Japan). In order to remove residual endotoxin contaminations, VT-1 was further passed through a Detoxi-Gel column (Wako Pure Chemical Industries (Osaka, Japan)). Percoll, Poly (dI–dC)·Poly (dI–dC), and oligonucleotides for the electrophoretic mobility shift assay (EMSA) were obtained from Amersham Biosciences (Uppsala, Sweden). Penicillin, streptomycin, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rabbit brain thromboplastin and bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO). MG132 was obtained from the Peptide Institute (Osaka, Japan), SB203580 and LY294002 from Carbiochem-Novabiochem (San Diego, CA), PD98059 from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA), and [γ^{32}P]ATP from Perkin Elmer Japan (Yokohama, Japan). Unless otherwise indicated, all other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Antibodies

Mouse monoclonal anti-human TF IgG_1 antibody (clone TF9-10H10) was obtained from American Diagnostica (Greenwich, CT). The rabbit polyclonal antibodies directed against human p65, p50, c-Rel, c-Fos, c-Jun, JunD, JunB, Egr-1, Egr-2, Sp3, Sp4, IκB kinase α/β (IKKα/β), IKKα, phospho-PI3-kinase (p85α) and Lyn, and the mouse monoclonal antibodies directed against human Sp1 and c-Src were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit polyclonal anti-PI3-kinase (p85α) was obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-phospho-IKKα/β, anti-phospho-p44/p42 MAPK (ERK1/2) and anti-ERK1/2 antibodies, mouse monoclonal anti-phospho-p38 MAPK (28B10) and anti-p38 MAPK (5F11) antibodies, and horse radish peroxidase (HRP)-labeled goat anti-rabbit IgG antibody were obtained from Cell Signaling Technology (Beverly, MA). HRP-labeled rabbit anti-mouse IgG antibody, fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse IgG antibody and mouse monoclonal IgG_1 were from DAKO (Glostrup, Denmark). Mouse monoclonal anti-c-Yes antibody (mAb1B7) was from Wako Pure Chemical Industries (Osaka Japan).

2.3. Cell culture and differentiation

To induce differentiation to macrophage-like THP-1 cells, human monocytic leukemia cell line THP-1 cells (1×10^6 cells/ml) obtained from the American Type Culture Collection (Rockville, MD) underwent treatment with 50 ng/ml PMA in RPMI 1640 supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), and 10% heat-inactivated fetal calf serum (FCS) (culture medium) for 48 h, as previously described [29]. The differentiated macrophage-like THP-1 cells were washed three times with cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS (−)) to remove undifferentiated cells and PMA, and then incubated with culture medium for 3 to 4 days with daily medium changes before use in the experiments. Human peripheral monocytes were isolated from blood collected from healthy adult volunteers by hypotonic density gradient centrifugation in Percoll [28]. All blood samples were obtained after informed consent. After removal of the non-adherent cells, the adherent monocytes were used in the experiments. Cell viability was determined by the MTT assay, as described previously [29].

2.4. TF procoagulant activity and immunohistochemical assay of TF antigen

TF procoagulant activity in the cells (24-well plates, 1.0×10^6 cells/well) was measured by a one-stage clotting assay, as described previously [20,29]. One unit of activity was defined as a clotting time of 20 s in a standard assay with

normal human plasma. The coagulant activity observed reflects the TF activity, since procoagulant activity was not detected in the cells when factor VII- or X-deficient plasma was used instead of normal plasma. An immunohistochemical assay for TF antigen was performed with mouse monoclonal anti-human TF IgG_1 or mouse monoclonal IgG_1, as previously described [29]. Fluorescence was detected by microscopy (Olympus IX70, Tokyo, Japan).

2.5. TF mRNA by the reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was prepared from the cells (24-well plate, 1.0×10^6 cells/well) using ISOGEN (Nippon Gene, Tokyo, Japan). Analysis of TF mRNA was performed by RT-PCR from the RNA preparation (1 μg/sample), as previously described [20,29]. After amplification and electrophoresis on 2% agarose gels, the gels were stained with 0.1 μg/ml ethidium bromide and photographed under UV light. The areas corresponding to the amplified cDNA on the gel were quantified by densitometric scanning using an Epi-Light FA500 (Aisin Cosmos Co., Aichi, Japan). In the range from 0.005 to 3.3 μg RNA, there was a linear relationship between the density obtained for each PCR product and the amount of total RNA subjected to RT-PCR.

2.6. EMSA and supershift assay

Nuclear extraction from the cells (3.0×10^6 cells/35-mm dish), EMSA and supershift assay were performed as described previously [29]. Briefly, the nuclear extracts were incubated with the following ^{32}P-labeled double-stranded oligonucleotide probes: TF-κB (NF-κB/Rel binding site in the TF promoter region), 5′-AGGGTCCCGGAGTTTCCTACCGGGA-3′; distal TF-AP-1 (AP-1 binding site in the TF promoter region), 5′-TCGGTGGCGCGGTTGAATCACTGG-3′; proximal TF-AP-1, 5′-GGTGAGTCATCCCTTGCAGGGTCC-3′; a prototypic Egr-1, 5′-CCCGGCGCGGGGGCGATTTCGAGTCA-3′; a prototypic Sp1, 5′-ATTCGATCGGGGCGGGGCGAGC-3′, with or without antibody against various transcription factors. The protein-oligonucleotide complexes (TF-κB- and TF-AP-1-oligonucleotide complexes) were separated from free oligonucleotides by electrophoresis in 4% polyacrylamide gels in 1× Tris borate EDTA (TBE) buffer. Egr-1- and Sp1-oligonucleotide complexes were separated in 0.5× TBE buffer. The gels were dried and radiolabeled bands were visualized by a BAS 2000-II Bio-Imaging Analyzer (Fuji Photo Film Co., Tokyo, Japan) or exposed to X-Omat AR film (Eastman Kodak, Rochester, NY).

2.7. Western blot analysis

Cells (3×10^6 cells) were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5 mM 4-(2-aminoethyl)-benzene sulfonyl-fluoride hydrochloride (AEBSF), 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μM E-64, 0.5 mM EDTA, 1 mM sodium orthovanadate). The cell lysates were boiled in Laemmli's sample buffer (LSB) and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, which was incubated with primary antibody of anti-phospho-PI3-kinase, anti-phospho-IKKα/β, anti-phospho-ERK1/2, anti-phospho-p38 MAPK, anti-PI3-kinase, anti-IKKα/β plus anti-IKKα, anti-ERK1/2, anti-p38 MAPK, anti-cYes, anti-c-Src, or anti-Lyn. After incubating with an HRP-labeled detection antibody, the signal was visualized by chemiluminescence (Pierce Chemical, Rockford, IL). Protein levels were quantified by densitometric analysis using NIH Image software.

2.8. Immunoprecipitation and kinase assay for Src family kinase

Immunoprecipitation and the kinase assay for the Src family kinases (c-Yes, c-Src, and Lyn) were performed after slight modification of a previously reported method [30]. VT-1-treated and untreated cells were washed twice with ice-cold PBS (−) and lysed in lysis buffer. The lysates (500 μg of cellular proteins) were incubated with 2 μg of anti-c-Yes, anti-c-Src, or anti-Lyn antibody for 1 h at 4 °C. The immune complexes were fixed on Protein G-Sepharose (KPL, Gaithersburg, MD) for 3 h with rotation at 4 °C, followed by washing two times in lysis buffer and two times in kinase buffer (10 mM Tris, pH 7.4, 10 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM sodium orthovanadate). The immunoprecipitates

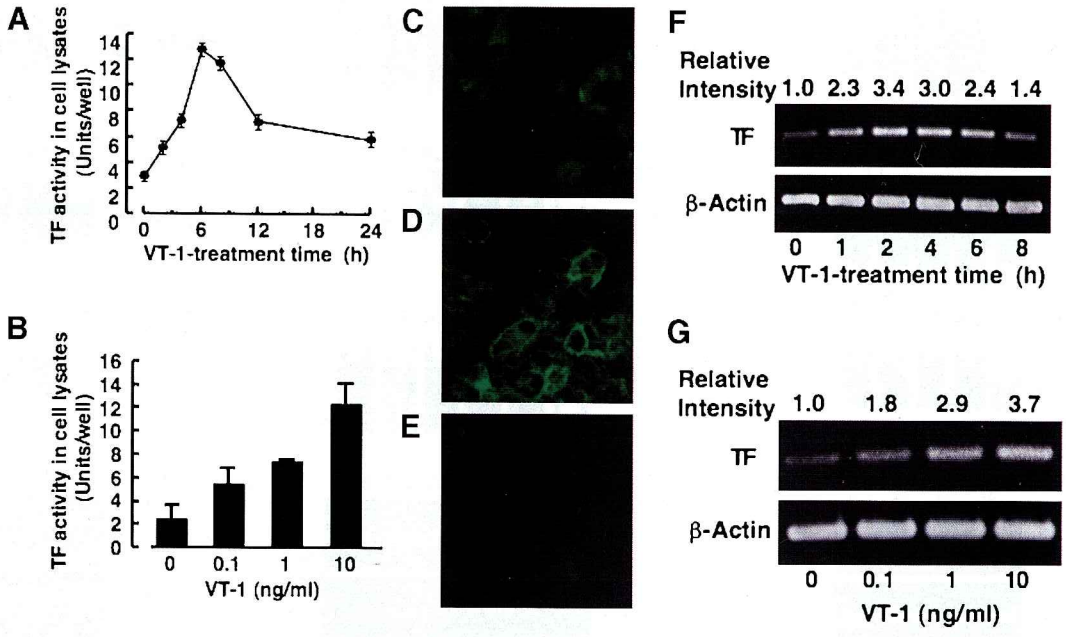

Fig. 1. Increase in TF activity, antigen and mRNA levels in macrophage-like THP-1 cells exposed to VT-1. (A, F) Macrophage-like THP-1 cells were treated with 10 ng/ml VT-1 for various times and then the procoagulant activity (A) and TF mRNA level (F) were determined. (B, G) After incubating with various concentrations of VT-1 (0–10 ng/ml) for 6 h (B) or 2 h (G), the procoagulant activity (B) and TF mRNA level (G) were determined. The data of TF activities represent the mean ± S.D. from four independent experiments. The TF mRNA levels were expressed as the relative intensity of control values (0 h or 0 ng/ml VT-1) after normalization to the β-actin mRNA levels. (C, D, E) The cells were incubated with (D, E) or without (C) 10 ng/ml VT-1 for 6 h. For immunohistochemical assays of TF antigen, VT-1-treated and untreated cells were incubated with anti-TF antibody and detected with FITC-labeled rabbit anti-mouse IgG antibody (C, D). The control experiment using mouse monoclonal IgG_1 was performed for VT-1-treated cells to assess nonspecific binding of FITC-labeled antibody (E). The data (C, D, E) represent a typical result of four independent experiments.

were used in an *in vitro* kinase reaction. Kinase assays were performed in the presence of 10 μM cold ATP, 1 μCi of [γ-^{32}P]ATP, and 5 μg of acid-denatured enolase/sample for 15 min at 25 °C. Reactions were stopped by boiling in LSB, and proteins were separated on SDS-PAGE (10% gels). The kinase activity for incorporation of [γ-^{32}P]ATP into enolase, c-Yes, c-Src, or Lyn was measured by autoradiography. The amount of c-Yes, c-Src, or Lyn applied for the kinase assay was estimated by Western blotting with anti-c-Yes, anti-c-Src, or anti-Lyn as the primary antibody.

2.9. c-Yes antisense oligonucleotide experiments

c-Yes phosphorothioate antisense (c-Yes AS) and its corresponding control randomized sequence phosphorothioate oligonucleotide (CS) were designed and manufactured by Biognostik (Gottinggen, Germany). These oligonucleotides were used according to the manufacturer's instructions. Macrophage-like THP-1 cells and peripheral adherent monocytes were exposed for 24 or 48 h to 4 μM of c-Yes AS or CS in RPMI containing 1% FCS. The c-Yes levels in the cell lysates were estimated by Western blotting.

3. Results

3.1. TF activity and mRNA levels in macrophage-like THP-1 cells treated with VT-1

At 24 h after treatment with 0.1 to 10 ng/ml VT-1, over 90% of the MTT reducing activity of the macrophage-like THP-1 cells was maintained as compared to untreated controls (data not shown). The effect of VT-1 on TF activity in differentiated macrophage-like THP-1 cells was time-dependent. Relative to untreated control cells, a 4.4-fold increase in TF activity was observed 6 h after treatment of the cells with 10 ng/ml VT-1, and the increased levels returned to a 2-fold level at 24 h (Fig. 1A). TF activity in cell lysates increased in a dose-dependent manner after treatment of the cells with 0.1 to 10 ng/ml VT-1 for 6 h (Fig. 1B). Up-regulation of TF expression on the cells treated with 10 ng/ml VT-1 was also observed by a semi-quantitative immunohistochemical assay using anti-TF antibody (Fig. 1C–E). LPS was not detected in the final VT-1 preparation, (<0.0005 EU/ml as determined by a Limulus test combined with chromogenic substrate), in addition, there was no increase in TF activity when differentiated macrophage-like THP-1 cells were treated with VT-1 previously boiled for 15 min. The results indicate that the cellular responses were independent of LPS. Relative to untreated control cells, a 3.4-fold increase in TF mRNA levels was observed 2 h after treatment of the macrophage-like THP-1 cells with 10 ng/ml VT-1, and the increased levels returned to a 1.4-fold level at 8 h (Fig. 1F). A concentration-dependent increase in TF mRNA levels was observed 2 h after treatment of the macrophage-like THP-1 cells with VT-1 (Fig. 1G). In contrast, no increases in TF activity, antigen or mRNA levels were observed in the undifferentiated THP-1 cells after stimulation with 10 ng/ml VT-1 for 2–24 h (data not shown).

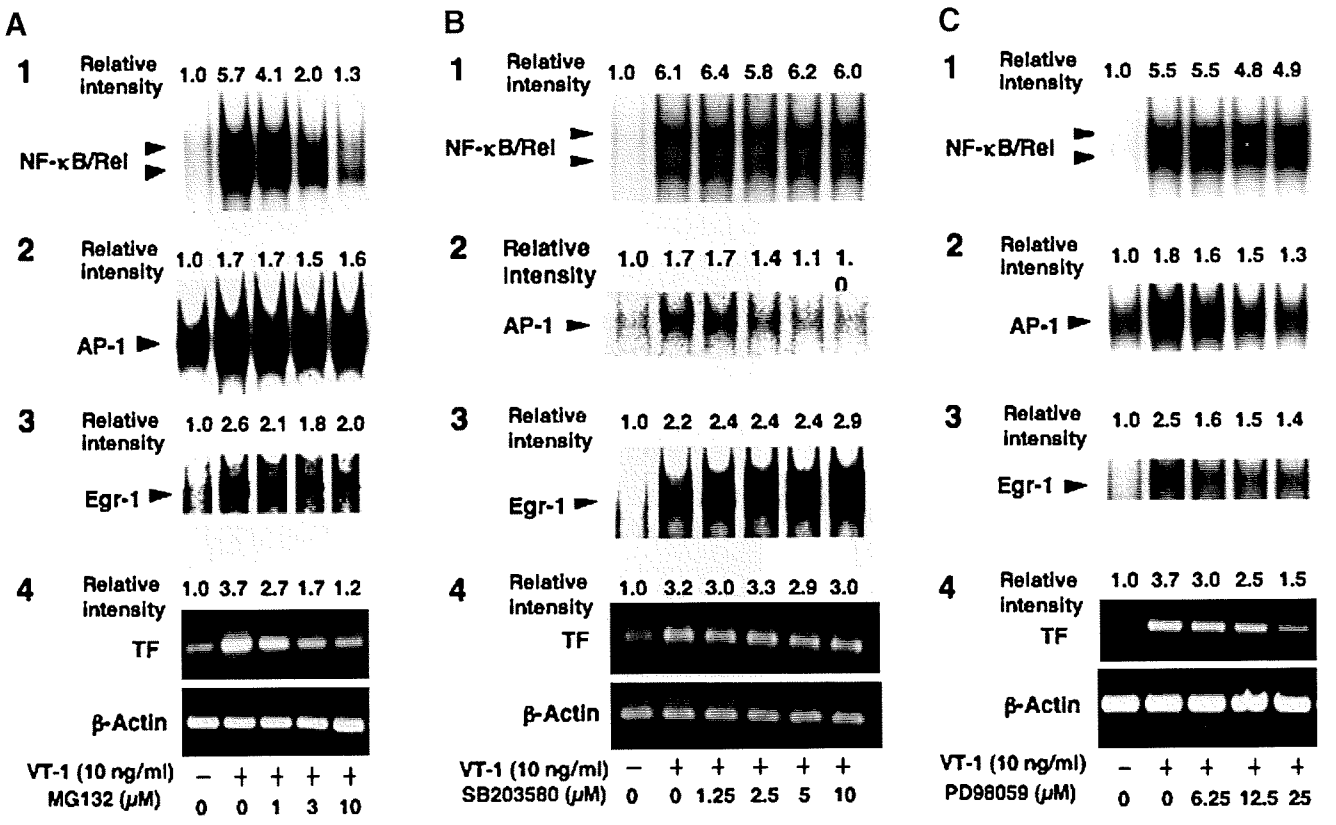

Fig. 2. Effects of inhibitors on VT-1-induced activation of NF-κB/Rel, AP-1, or Egr-1, and on the up-regulation of TF mRNA levels in macrophage-like THP-1 cells. (A, B, C) Macrophage-like THP-1 cells were pretreated with or without various concentrations of MG132, SB203580 or PD98059 for 1 h before stimulation of 10 ng/ml VT-1 for 2 h. The nuclear proteins extracted from the cells were incubated with ^{32}P-labeled TF- κB (A-1, B-1, C-1), proximal TF-AP-1 (A-2, B-2, C-2), or Egr-1 (A-3, B-3, C-3) oligonucleotide. EMSAs were performed according to the method described in Materials and methods. TF mRNA levels were determined by RT-PCR (A-4, B-4, C-4). The data represent a typical result and the average relative intensity from three independent experiments.

3.2. VT-1-induced activation of transcription factors and effect of inhibitors on their activations

The human TF promoter gene contains binding sites for NF-κB/Rel, AP-1, Egr-1 and Sp1 [31]. Activation, nuclear translocation and composition of these transcription factors in VT-1-treated macrophage-like THP-1 cells were examined by EMSA and supershift assays. The results of these assays indicated that the NF-κB molecules bound to TF-κB oligo had two bands that consisted of p65 and p50 in the lower band and p65 and c-Rel in the upper band as a previous study [29]. Additionally, VT-1 activated the AP-1 families including c-Jun, JunD and c-Fos, and Egr-1 leading to translocation into the nuclei (data not shown). In contrast, Sp1, Sp3 and Sp4 were constitutively activated in the cells and were not up-regulated by VT-1 stimulation (data not shown). Therefore, we decided to examine the effect of inhibitors on VT-1-induced NF-κB/Rel binding to TF-κB oligo, AP-1 binding to proximal TF-AP-1 oligo, and Egr-1 binding to Egr-1 oligo (Fig. 2).

In a dose-dependent manner and in parallel with the TF mRNA levels, VT-1-induced NF-κB/Rel activation was inhibited by pretreatment of the cells with the proteasome inhibitor MG132 (Fig. 2A-1 and A-4). However, the VT-1-induced AP-1 and Egr-1 activations were not influenced by the MG132 pretreatment (Figs. 2A-2 and 3). When cells were pretreated with SB203580, an inhibitor for p38 MAPK, VT-1-induced AP-1 activation was inhibited in a dose-dependent manner (Fig. 2B-2), but this inhibitor did not affect either the NF-κB/Rel and Egr-1 activations or the increased TF mRNA levels (Figs. 2B-1, 3 and 4). Pretreatment of the cells with PD98059, an inhibitor of the mitogen-activated protein kinase kinase (MEK)/ERK pathway, inhibited the AP-1 and Egr-1 activations and the increase in the TF mRNA levels in VT-1-stimulated cells in a dose-dependent manner (Figs. 2C-2, 3 and 4); however, there was no change noted for the NF-κB/Rel activations (Fig. 2C-1). These results indicate that the VT-1-induced up-regulation of TF mRNA levels occurred in parallel with the activation of the proteasome/NF-κB/Rel and MEK/ERK1/2/Egr-1 pathways, but not the p38 MAPK/AP-1 pathway.

To further examine the activation of IKKα/β and ERK1/2 in the VT-1-treated cells, Western blotting was used to detect phosphorylation of these kinases. Phosphorylation of IKKβ, ERK2 and p38 MAPK, but not IKKα and ERK1, was observed in the cells beginning 15 min after the VT-1 stimulation (Fig. 3A). Additionally, phosphorylation of PI3-kinase was transiently observed in the cells at 3 min after VT-1 stimulation (Fig. 3B). Pretreatment of the cells with increasing concentrations of the PI3-kinase inhibitor, LY294002, inhibited the up-regulation of TF mRNA levels induced by VT-1 (Fig. 3C) and the phosphorylations of IKKβ and ERK2 (Fig. 3D).

3.3. Involvement of c-Yes in intracellular signal pathways in the VT-1-induced up-regulation of TF-mRNA and activity levels

The activation of Src family kinases including c-Yes, c-Src and Lyn were analyzed in macrophage-like THP-1 cells treated with or without 10 ng/ml VT-1 (Fig. 4). An approximate 2-fold increase in the phosphorylation of the enolase substrate by the c-

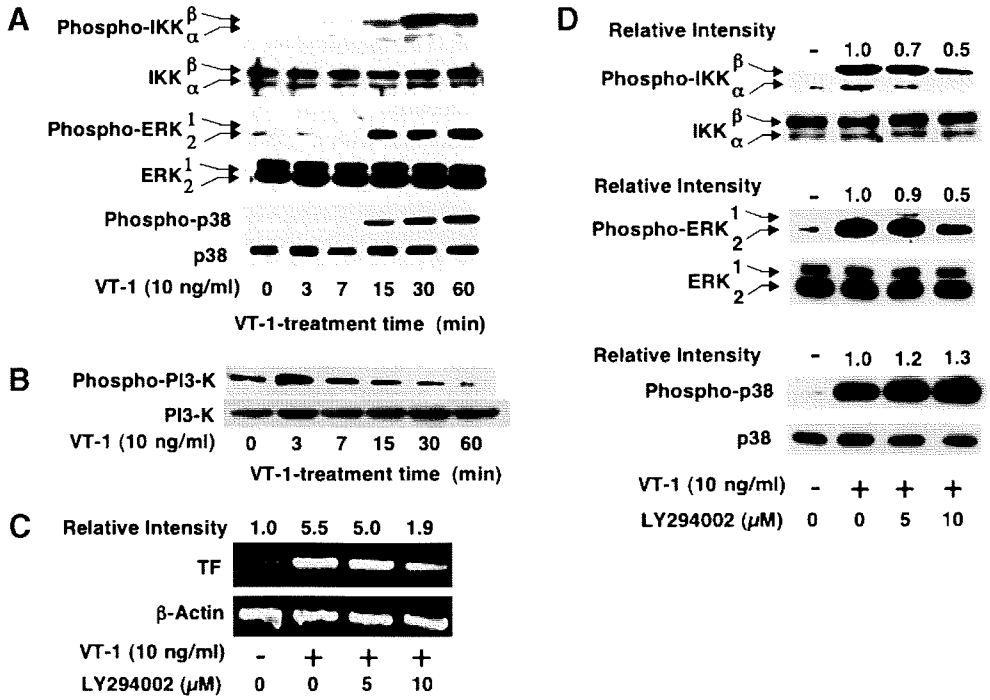

Fig. 3. Effects of inhibitors on VT-1-induced phosphorylation of IKKα/β, ERK1/2, p38 MAPK and PI3-kinase in macrophage-like THP-1 cells. (A, B) Phosphorylation of IKKα/β, ERK1/2, p38 MAPK and PI3-kinase in the cell lysates prepared from the cells treated with 10 ng/ml VT-1 for indicated times were analyzed by Western blotting. (C) The cells were pretreated with LY294002 (0, 5, 10 μM) for 30 min before stimulation of 10 ng/ml VT-1 for 2 h. TF mRNA levels were determined by RT-PCR. (D) The cells were pretreated with LY294002 (0, 5, 10 μM) for 30 min before stimulation of 10 ng/ml VT-1 for 1 h. The phosphorylation of their kinases was analyzed by Western blotting. The data represent a typical result and the average relative intensity from three independent experiments.

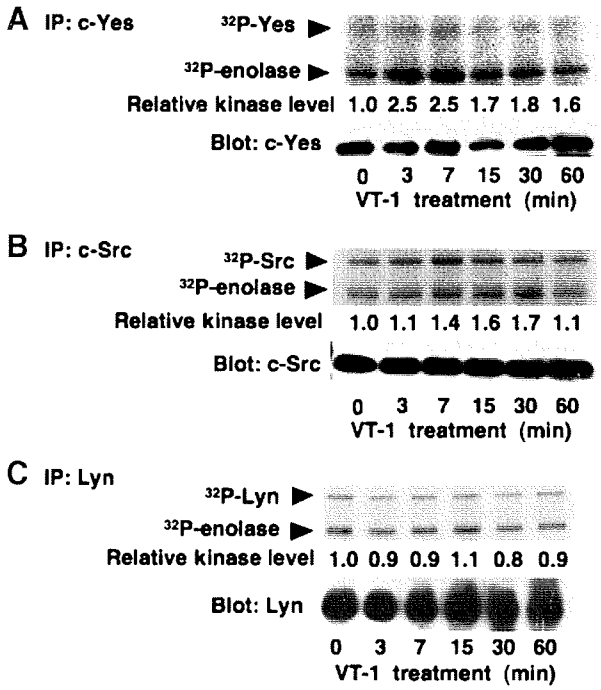

Fig. 4. Kinetic analysis of Src family kinase activities in macrophage-like THP-1 cells treated with VT-1. Macrophage-like THP-1 cells incubated with or without VT-1 (10 ng/ml) were lysed at the indicated times. Src family kinase c-Yes (A), c-Src (B), and Lyn (C) were isolated from each lysate by immunoprecipitation with anti-c-Yes, anti-c-Src or anti-Lyn antibody and subjected to an *in vitro* kinase assay. $^{32}PO_4$-incorporated c-Yes, c-Src, Lyn and enolase were separated by SDS-PAGE and exposed to X-ray film. The amount of immunoprecipitated c-Yes, c-Src, or Lyn protein was assayed by Western blotting with anti-c-Yes, anti-c-Src, or anti-Lyn. The data represent a typical result and the average relative kinase activity to phosphorylate enolase from three independent experiments.

Yes kinase was observed in cells treated with VT-1 for 3 and 7 min (Fig. 4A). The kinase activity of c-Src also slightly increased in VT-1-treated cells, although peak activity was not observed until 30 min after the stimulation (Fig. 4B). No increase in the activity of Lyn was observed in the VT-1-treated cells (Fig. 4C).

In order to evaluate involvement of c-Yes in the VT-1-induced up-regulation of the TF transcription and intracellular signaling pathway after c-Yes activation, an antisense (AS) experiment was conducted. Macrophage-like THP-1 cells treated with specific c-Yes AS for 48 h exhibited a 90% decrease in c-Yes expression relative to control cells, while treatment with a random control sense oligo (CS) had no effect (Fig. 5A). In addition, phosphorylation of PI3-kinase, IKKβ and ERK2 observed in the VT-1-treated control cells was attenuated in the c-Yes knockdown cells, but not in the c-Yes CS-inserted cells, similar to that observed for c-Yes expression (Fig. 5B, C and D). In contrast, the VT-1-induced phosphorylation of p38 MAPK observed in the control cells was not influenced in the c-Yes knockdown and c-Yes CS-inserted cells (Fig. 5E). Activations of NF-κB/Rel (Fig. 5F) and Egr-1 (Fig. 5G) in VT-1-stimulated cells were also reduced in the c-Yes knockdown cells, but not in the c-Yes CS cells, with parallel decreases noted for both TF mRNA levels (Fig. 6A) and activity (Fig. 6B). These results indicate, at least in part, that c-Yes is essential for up-regulation of TF transcription in VT-1 stimulated macrophage-like THP-1 cells and that c-Yes tyrosine kinase activity participates in the phosphorylation of PI-3-kinase, IKKβ and ERK2 and in the activation of the NF-κB/Rel and Egr-1 pathways. We also investigated the participation of c-Yes on VT-1-induced TF up-regulation in monocytes, by constructing c-Yes knockdown cells of adherent monocytes that were isolated from circulating plasma (Fig. 6C and D). Similar to macrophage-like THP-1 cells, the adherent monocytes up-regulated TF mRNA and activity levels in response to VT-1, with the up-regulation attenuated after insertion of the c-Yes AS into the cells.

4. Discussion

The present work examined TF expression on differentiated macrophage-like THP-1 cells, which are widely utilized as adherent macrophage substitutes, after being exposed to VT-1. After exposure, there was a transient up-regulation of both TF activity and expression with increased levels of TF mRNA along with the retention of cell viability.

The present study was designed to further investigate the signaling pathways to induce TF up-regulation in the cells exposed to VT-1. Results indicated that NF-κB/Rel molecules, AP-1 families, and Egr-1 were activated by VT-1 followed by translocation into the nuclei. NF-κB/Rel activation is mediated by proteasomal degradation of IκB phosphorylated by phosphoactivated IKKα/β [32]. The present results indicated that IKKβ, but not IKKα, was predominantly phosphorylated in the VT-1-stimulated cells and that pretreatment of the cells with MG132 inhibited the VT-1-induced up-regulation of TF mRNA levels in parallel with the decreased NF-κB/Rel activation. Recently, it was reported that activation of NF-κB/Rel to associate with a selective regulation of major pro-inflammatory and prothrombotic mediators including TNF-α and TF is dependent on phosphoactivated IKKβ, but not on IKKα [33]. The current results suggest that VT-1-induced TF transcriptional up-regulation was mediated at least in part by the IKKβ/proteasome/NF-κB/Rel pathway.

AP-1 activation is mediated by p38 MAPK, ERKs and/or c-Jun N-terminal kinase/stress-activated protein kinases [34]. In the present experiments, phosphorylation of p38 MAPK occurred after VT-1 treatment of the cells, and pretreatment with SB203580, a p38 MAPK inhibitor, abolished this VT-1-induced AP-1 activation. However, up-regulation of TF transcription was never inhibited, which suggests that AP-1 activation did not directly participate in the up-regulation of TF mRNA levels.

Egr-1 activation is required for maximal induction of the TNF-α and TF genes in monocytic cells stimulated with LPS [35]. Egr-1 is activated by phosphoactivated ERK1/2 [36]. It has been reported that hypoxia-induced Egr-1 activation in endothelial cells [37] and the toxin Malachite Green-induced malignant transformation in hamster embryo cells is dependent on phosphoactivation of ERK2, but not ERK1 [38], suggesting that there is a distinct phosphoactivation of ERKs in various

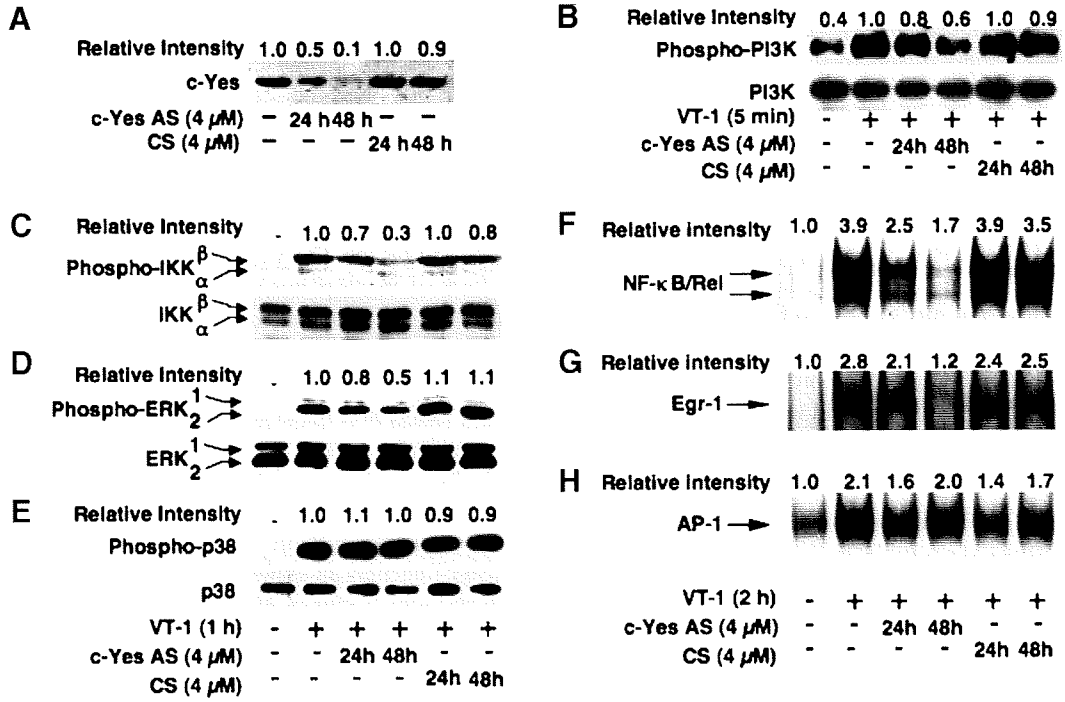

Fig. 5. Effect of c-Yes knockdown on VT-1-induced phosphorylation of kinases and activation of TF-κB/Rel, Egr-1 and AP-1 in macrophage-like THP-1 cells. (A) Macrophage-like THP-1 cells were incubated with or without specific c-Yes antisense (c-Yes AS) or random control sense (CS) oligonucleotide (4 μM) for 24 or 48 h. The cells were solubilized and c-Yes protein in cell lysates was analyzed by Western blotting. (B, C, D, E, F, G, H) The cells treated with c-Yes AS or CS were further incubated with or without 10 ng/ml VT-1 for 5 min (B), 1 h (C–E) or 2 h (F–H). The cell lysates and nuclear proteins extracted from the cells were analyzed by Western blotting (B–E) and by EMSA (F–H). The data represent a typical result and the average relative intensity from three independent experiments.

cells with various stimuli and thus ERK2 has a role in Egr-1 activation [39]. The present results indicated that ERK2 was predominantly phosphorylated by VT-1 treatment of the cells and that PD98059 pretreatment of the cells inhibited the VT-1-induced up-regulation of the TF mRNA levels in a parallel with Egr-1 inhibition. This suggests that the VT-1-induced up-regulation of TF transcription may be dependent upon the MEK/ERK2/Egr-1 pathway.

The VT-1 receptor, Gb3, is a glycosphingolipid (GSL). GSLs are known to form microdomains containing cholesterol at the

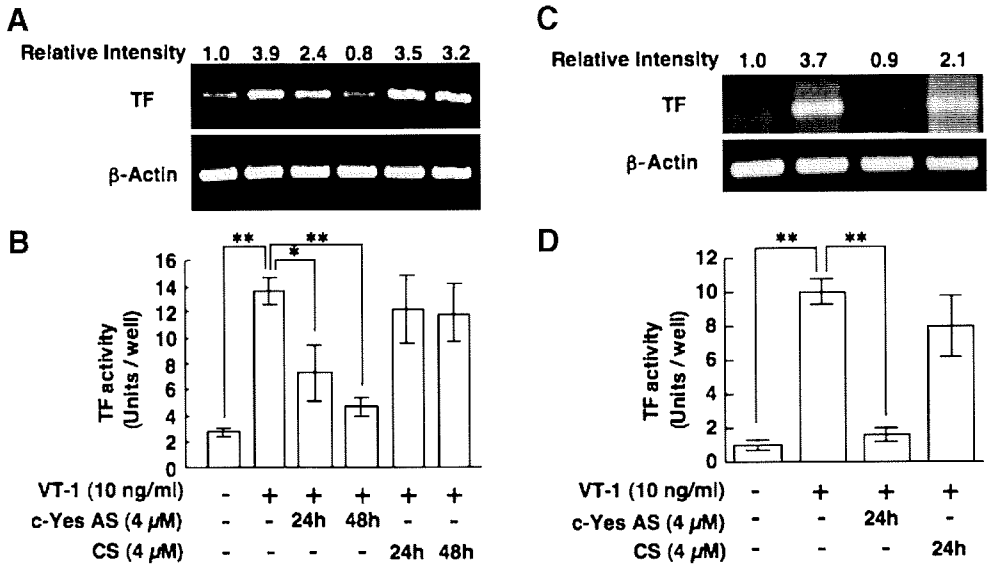

Fig. 6. Effect of c-Yes knockdown on VT-1-induced TF mRNA levels and TF activities in macrophage-like THP-1 cells and adherent monocytes. (A, B) Macrophage-like THP-1 cells (1×10^6 cells/well) were incubated with or without c-Yes AS or CS oligonucleotide (4 μM) for 24 or 48 h. (C, D) Adherent monocytes (0.65×10^6 cells/well) were incubated with or without c-Yes AS or CS oligonucleotide (4 μM) for 24 or 48 h. The cells were further incubated with or without 10 ng/ml VT-1 for 2 h (A, C) or 6 h (B, D). The data represent a typical result and the average relative intensity (A, C), and the TF procoagulant activities expressed as the mean±S.D. (B, D) from four independent experiments. The statistical differences were analyzed by Student's *t*-tests; *<0.05, **<0.01.

surface of the plasma membranes. Since the GSL microdomains are associated with a variety of signaling molecules, such as the Src family kinases, they are postulated to be involved in signal transduction. It has been reported that VT-1 bound to Gb3 on the surface of the human renal tubular-derived ACHN cell line leads to the association of Gb3 with c-Yes [13]. In the present study, it was observed that within 3 min of VT-1 stimulation of the macrophage-like THP-1 cells, c-Yes tyrosine kinase was quickly activated and within 30 min, was followed by a slow activation of c-Src without activation of Lyn. Therefore, we constructed c-Yes knockdown macrophage-like THP-1 cells to evaluate the participation of c-Yes in the up-regulation of TF by VT-1.

The present work revealed that PI3-kinase was quickly phosphorylated after VT-1 stimulation of macrophage-like THP-1 cells and that the VT-1-induced phosphorylations of IKKβ and ERK2 along with the up-regulation of TF transcription were inhibited by pretreatment of the cells with the PI3-kinase inhibitor, LY294002. Furthermore, phosphorylations of PI3-kinase, IKKβ and ERK2, activations of NF-κB/Rel and Egr-1, and increased TF mRNA and activity levels observed in VT-1-treated control cells were suppressed by the c-Yes knockdown in a parallel with the reduced expression of c-Yes. However, phosphorylation of p38 MAPK and activation of AP-1 in the VT-1-treated control cells was not affected by c-Yes knockdown or LY294002 treatment. Overall, the present results suggest that, at least in part, VT-1 stimulation activated c-Yes and the activated c-Yes up-regulated TF transcription through activations of the PI3-kinase/IKKβ/proteasome/NF-κB/Rel and PI3-kinase/MEK/ERK2/Egr-1 pathways. This is the first report of a protein whose expression is controlled by c-Yes activated by VT-1 stimulation and TF expression that is mediated by activated c-Yes. On the other hand, a slow and slight activation of c-Src was observed in the present work. Therefore, with the exception of Lyn, we cannot rule out participation of Src family kinases other than c-Yes in the VT-1 induction of TF transcription.

We further constructed c-Yes knockdown adherent monocytes and observed that these knockdown monocytes also suppressed the increase in TF mRNA and the activity levels observed in VT-1-treated control cells. This indicates that VT-1-induced TF up-regulation in adherent monocytes, as well as macrophages, could possibly be regulated by c-Yes activation. The cytokine Oncostatin M has been found to activate the tyrosine phosphorylation signal transduction pathway via the activation of c-Yes in endothelial cells [40] and also induces increased synthesis of TF in human vascular smooth muscle cells [41]. Activated Src family proteins, including c-Yes and p60src, activate PI3-kinase [42], and the activated PI3-kinase activates Egr-1 through ERK1/2 activation, which then induces NF-κB activation through the activation of the PI3-kinase/Akt/IκB kinase pathway [43,44]. However, studies on the role of the PI3-kinase/Akt pathway in NF-κB- and ERK1/2-dependent gene expression are controversial. It has been reported that inhibition of the PI3-kinase/Akt pathway by pretreating monocytes with LY294002 enhances LPS-induced up-regulation of TF mRNA levels through Egr-1 and NF-κB activation via activation of ERK1/2 and enhanced nuclear translocation of p65, respectively [45]. In preliminary experiments, we observed that LPS-induced up-regulation of TF in macrophage-like THP-1 cells was not attenuated in the c-Yes knockdown cells (data not shown). This implies that the up-regulation may not be mediated by c-Yes activation, which is in contrast to the effects observed for VT-1 on TF expression.

In addition to the VT-1-induced up-regulation of TF activity that is demonstrated in the present work, it has also recently been reported that the TF–VIIa–Xa ternary complex formed on the surface of macrophage/monocyte lineage and/or released Xa induces production of inflammatory cytokines such as IL-1, 6 and 8 via PAR1 and 2 activation [16,46,47]. These cytokines also induce TF expression on macrophage/monocytes [19,20]. Thus, VT-1-stimulated macrophage/monocyte lineage may additively or synergistically amplify TF overexpression on cells through the stimulation of autocrine inflammatory cytokine responses. During the coagulation–inflammation–thrombosis circuit, overexpression of TF on the macrophage/monocyte lineage may play an important role in the development of disease complications, such as HUS and CNS vascular damage.

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