10% heat-denatured FBS (HyClone, Logan, UT), 500 μg/ml heparin (Sigma-Aldrich), and 2 ng/ml human acidic fibroblast growth factor (FGF; PeproTech, London, U.K.) in dishes treated with 0.1% gelatin (Wako Pure Chemical, Osaka, Japan). Cells were cultured at 37°C in an atmosphere of 5% CO_2, and routinely passaged by trypsinization when achieving confluence. HUVECs used in all experiments were of up to seven passages.

Transfection was performed using Effectene Transfection Reagent (Qiagen, Hilden, Germany). Overexpression of *ID* genes in HUVECs was achieved using pBLAST49-hId1a(c) and pBLAST49-hId3(c) plasmid vectors (InvivoGen, San Diego, CA). pBLAST49-mcs (InvivoGen) was used as a negative control.

To knockdown *ID1* and *ID3*, small hairpin RNAs (shRNAs) were designed. The sequences of *ID1*- and *ID3* shRNAs were as follows: TCCCAAAGAATCATGAAAGTCGCCAGTTCAAGAGACTGGCGACTTTCATGATTCTTTT and TCGGATCCAACCTCACAGCACCTCACTTCTTCAAGAGAGAAGTGAGGTGCTGTGAGGTTTTTTTTGGAAAAGCTTGG, respectively, with 3′ single-strand overhangs for ligation into RNA expression vectors (psiRNA-hH1 neo (InvivoGen) for Id1; pSilencer 2.0-U6 (Ambion, Austin, TX) for Id3) containing H1 or U6 RNA polymerase III promoter.

Transfection efficiency was examined using X-Gal staining assay (Gene Therapy Systems, San Diego, CA). LacZ expression vector was transfected into HUVECs under the same conditions in which Id3 transfectants and Id1/Id3 RNAi transfectants were generated. After incubation for 24 h, the cells were placed in the fixing buffer for 15 min at room temperature. Then, X-Gal staining solution was added to the dishes, and the cells were incubated for 10 h at 37°C. After incubation, stained and unstained cells were counted. The transfection efficiency was determined to be 62.6 ± 9.6%.

Endotoxin levels in the vector preparations were measured using endotoxin detection kit based on *Limulus* amebocyte lysate assay (Endospecy ES-50M; Seikagaku Corporation, Tokyo, Japan). Contamination of endotoxin was undetectable.

RT-PCR

Total RNA was isolated from the cells using RNeasy Mini kit (Qiagen), and was treated with DNase inhibitor (DNA-free (Ambion)). First-strand cDNAs were synthesized from RNA using ImProm-II reverse transcription system (Promega, Madison, WI). Quantitative RT-PCR was performed using a real-time RT-PCR machine (LightCycler), with LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) and gene-specific primer sets. The data were analyzed by fit-points method using the LightCycler analysis software. β-Actin or GAPDH were used to normalize total RNA levels. The assays were done in triplicate.

Induction of Id1 and Id3 in HUVECs after stimulation for 24 h by VEGF (20 ng/ml), TGFβ (0.5 ng/ml), TNF-α (20 ng/ml), or IL-1β (1 ng/ml) (all from PeproTech) was examined by conventional RT-PCR. The PCR products were electrophoresed, stained with SYBR Gold (Molecular Probes, Eugene, OR), and relative mRNA levels were calculated from the band intensity using β-actin as a reference.

Because the ordinary culture medium for HUVEC contained acidic FGF, the effect of basic FGF (bFGF; PeproTech) for the induction of Id1 and Id3 was examined under a different culture condition. HUVECs were cultured in serum-free medium (Human Endothelial-SFM; Invitrogen Life Technologies, Carlsbad, CA) supplemented with epidermal growth factor (10 ng/ml; Invitrogen Life Technologies) in accordance with the manufacturer's instruction, in the presence or absence of bFGF (10 ng/ml). After 72 h, Id1 and Id3 mRNA levels were quantitated as described above.

The primer pairs used for RT-PCR were the following: Id1, 5′-AGCCAGTCCGCCAAGAATCAT-3′(forward),5′-ACTCACTCCCCAGCATGAAG-3′ (reverse); Id3, 5′-CTCCACGCTCTGAAAAGACC-3′ (forward), 5′-ACTCAGATTAAGCCAGGTGGA-3′ (reverse); $p16^{INK4a}$, 5′-AGCCTTCGGCTGACTGGCTGG-3′ (forward), 5′-GCAGTTAAGGGGGCACGAGTG-3′ (reverse); ICAM-1, 5′-ACCTGGCAATGCCCAGACATCTGTGT-3′ (forward), 5′-GTACACGGTGAGGAAGGTTTAGCTGTTG-3′ (reverse); E-selectin, 5′-AAAACTTCCATGAGGCCAAA-3′ (forward), 5′-GCATTCCTCTCTTCCAGAGC-3′ (reverse); matrix metalloproteinase (MMP)2, 5′-ATGACAGCTGCACCACTGAG-3′ (forward), 5′-TGATGTCATCCTGGGACAGA-3′ (reverse); and MMP9, 5′-GGCGCTCATGTACCCTATGT-3′ (forward), 5′-CCCTCAGTGAAGCGGTACAT-3′ (reverse).

Cell proliferation assay

The proliferative activity of HUVECs was measured using the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan). Cells were cultured on gelatin-coated flat-bottom 96-well microtiter plate at 2×10^4 cells/well. After 24 h, 10 μl of WST-1 solution was added to each well, and the cells were incubated at 37°C in an atmosphere of 5% CO_2 for 4 h. The supernatant solutions were transferred to a new 96-well plate, and the absorbance of each well was measured at 480 nm. Experiments were performed in triplicate, and the proliferative activity was calculated as the mean ± SD of the triplicate wells divided by that of the controls.

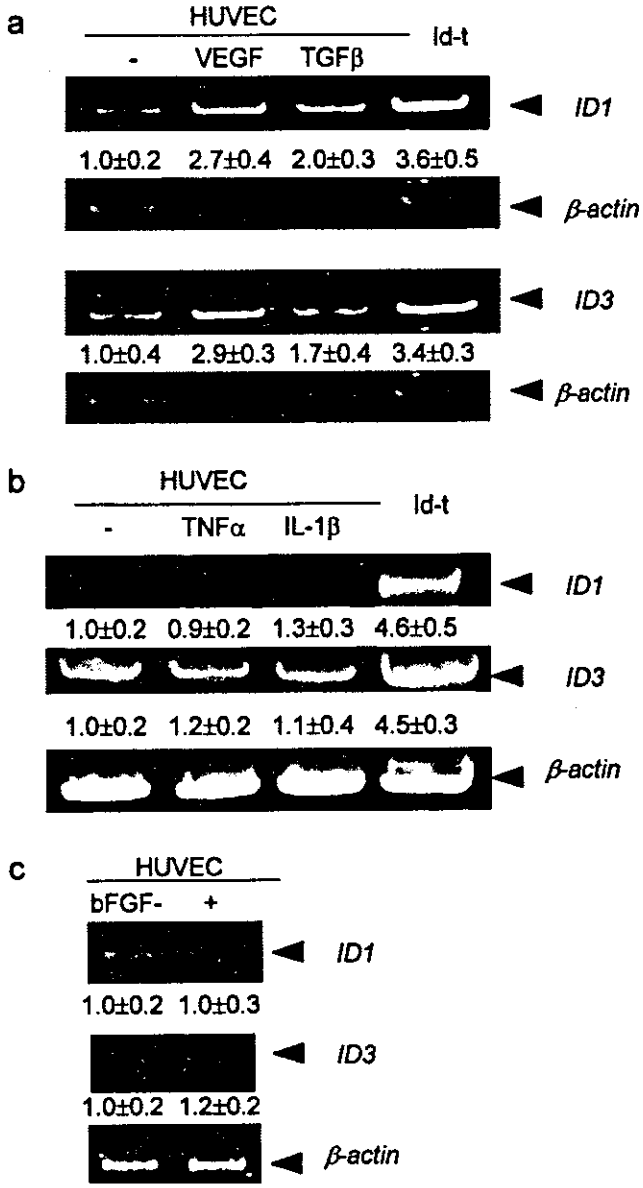

FIGURE 1. Expression of *ID1* and *ID3* mRNA is induced in HUVECs by VEGF or TGFβ. *a* and *b*, Id1 and Id3 mRNA levels were measured in HUVECs with or without stimulation with VEGF (20 ng/ml), TGFβ (0.5 ng/ml) (*a*), TNF-α (20 ng/ml), or IL-1β (1 ng/ml) (*b*) for 24 h. HUVECs transfected with *ID1* (Id1-t) or *ID3* (Id3-t) were used as the positive controls. *c*, In the case of bFGF, to exclude influence from acidic FGF contained in the ordinary culture medium, HUVECs were cultured in serum-free medium supplemented with epidermal growth factor for 72 h, in the presence or absence of bFGF (10 ng/ml). The densitometric intensity of each band was normalized to β-actin, and intensities relative to the control cells are shown under each panel.

Apoptosis induction

Apoptosis was induced in Id1-, Id3-, or mock-transfected HUVECs by serum deprivation (22). After the cells were cultured without FBS for 48 h, apoptotic cells were detected with Annexin V-FITC staining (Annexin V-FITC kit; Beckman Coulter). Freshly split HUVECs with or without treatment with agonistic anti-Fas/CD95 Ab (7C11; Beckman Coulter) were used as positive and negative controls, respectively.

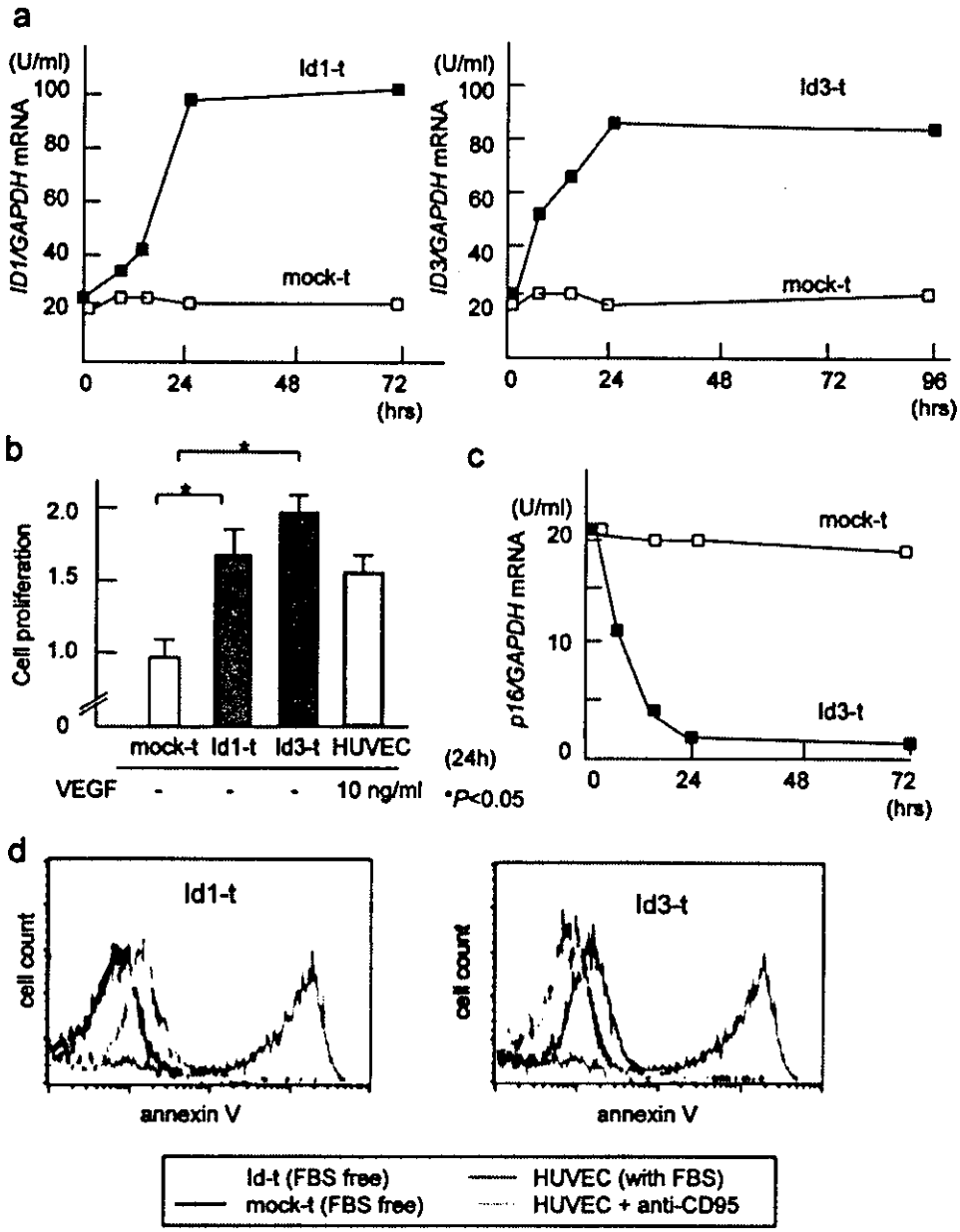

FIGURE 2. Overexpression of *ID1* or *ID3* induces proliferation of HUVECs. *a*, pBLAST49-hId1a(c) (Id1-t), pBLAST49-hId3(c) (Id3-t), or pBLAST49-mcs (mock-t) vector was transfected into HUVECs. Total RNA was isolated from each transfectant, and Id1 or Id3 mRNA level was sequentially quantitated using real-time RT-PCR. *b*, Proliferation of HUVECs at 24 h after transfection. Id1-t and Id3-t exhibited significantly higher proliferation in the absence of VEGF, compared with mock transfectants. Proliferation of Id transfectants was comparable with that of untransfected HUVECs stimulated with 10 ng/ml VEGF. Proliferation was measured using WST-1 assay. *c*, $p16^{INK4a}$ mRNA levels of *ID3* and mock transfectants were measured with real-time RT-PCR. $p16^{INK4a}$ mRNA expression was inversely correlated with Id3 mRNA shown in *a*. *d*, Apoptosis was induced in Id1, Id3, or mock transfectants by serum deprivation for 48 h, and the cells were stained with Annexin V-FITC. Freshly split HUVECs with or without treatment with agonistic anti-Fas/CD95 Ab (7C11) were used as positive and negative controls, respectively. Apoptosis induction was inhibited by overexpression of Id3, but not by Id1.

Flow cytometry

HUVECs were harvested by washing with 0.02% EDTA-PBS followed by treatment with trypsin-EDTA (InvivoGen), resuspended in PBS containing 0.1% BSA (Sigma-Aldrich) and 0.1% sodium azide, and incubated for 30 min with 50 ng/ml human γ-globulin (Sigma-Aldrich) at room temperature, to block for nonspecific binding.

For direct immunofluorescence staining, the cells were incubated with fluorescence-labeled mAbs or the isotype-matched controls for 30 min on ice, and then washed three times using a washing buffer consisting of PBS containing 0.2% BSA and 0.1% sodium azide. For indirect immunofluorescence staining, the cells were incubated with unlabeled primary mAbs or the isotype-matched Abs for 30 min on ice, washed with washing buffer, and then incubated with PE-conjugated goat anti-mouse IgG (Beckman Coulter), followed by another washing in washing buffer. Fluorescence intensity was analyzed in the EPICS XL (Beckman Coulter) or in the FACSCalibur (BD Biosciences).

Transmigration assay

Transmigration assays were performed using 8-μm-pore Transwell chambers (Corning, Corning, NY). HUVECs harvested by trypsinization were resuspended in MCDB151 medium containing 0.2% BSA, and seeded at 1×10^4 cells per well on the gelatin-coated upper chambers. Lower chambers were filled with 600 μl of the same medium supplemented with 10% FBS without or with either VEGF (20 ng/ml) or IL-1β (0.5 ng/ml). After incubation for 8 h at 37°C, 60 μl of 0.2% EDTA-PBS was added to the lower wells, and cells were harvested. The number of cells in the region corresponding to endothelial cells was counted in the EPICS, after acquiring cells in each sample for 50 s. The assays were done in triplicate.

Zymography

HUVECs were seeded on uncoated 100-mm dishes at 1×10^6 cells/well and were incubated with a 1:1 mixture of serum-free DMEM and F-12 Ham's medium (Sigma-Aldrich) for 12 h at 37°C. Then, supernatants were collected and, after removal of cells by centrifugation, were concentrated using the Centricon YM-10 concentrator (Millipore, Bedford, MA). Samples were then diluted with the same volume of sample buffer, and the MMPs were separated in SDS-PAGE using 10% polyacrylamide gels containing 0.1% gelatin. The gels were then incubated in 6.25% Triton X-100 (Wako Pure Chemical) for 1 h to remove SDS, and then placed for overnight in an incubation buffer containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ for development of enzyme activity. The gels were stained using Coomassie brilliant blue and destained in methanol/acetic acid. Gelatinase activity was detected as unstained bands.

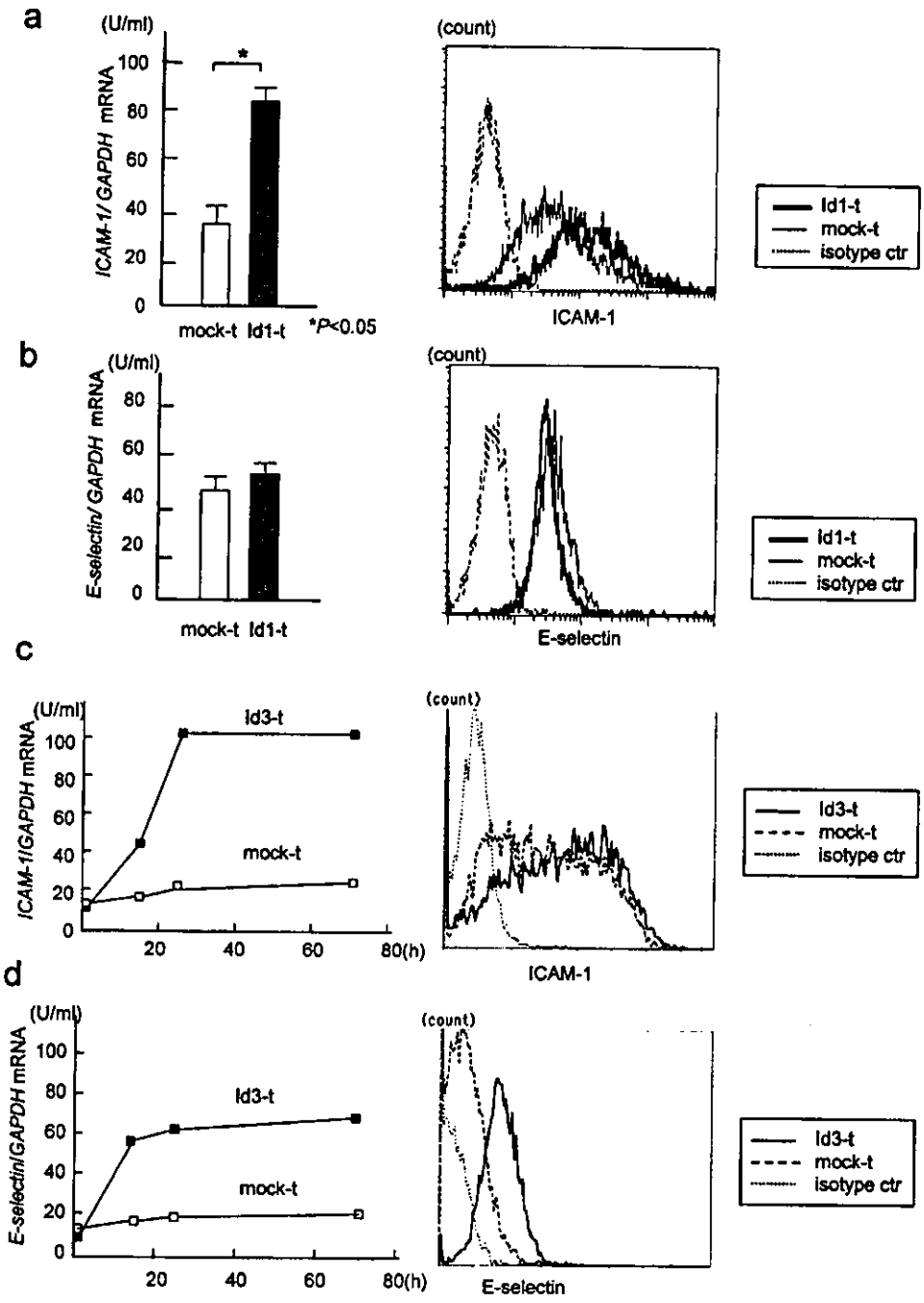

FIGURE 3. Overexpression of Id1 or Id3 induces activation of HUVECs. *a* and *b*, mRNA levels and surface expression of ICAM-1 (*a*) and E-selectin (*b*) in Id1 transfectants at 24 h after transfection. *c* and *d*, Kinetics of ICAM-1 (*c*) and E-selectin (*d*) mRNA levels in Id3 transfectants (*left*) and surface expression at 24 h. Significant up-regulation of ICAM-1 was observed in both transfectants, whereas expression of E-selectin was up-regulated only in Id3 transfectants.

In vitro tube formation

In vitro tube formation assay was performed using the Matrigel basement membrane matrix (BD Biosciences). Matrigel, kept on ice, was placed at 1 ml/well in six-well culture plates. The plates were then incubated at 37°C for 30 min to allow Matrigel to solidify. HUVECs were seeded at 5×10^4 cells per well on the top of the solidified Matrigel in the presence or absence of VEGF, and the plate was incubated at 37°C for 24 h. Tube formation on Matrigel was observed and analyzed under the microscope. The degree of angiogenesis was measured by multiplying the number of branch points by the total number of branches (23).

Statistics

Statistical significance was analyzed with Student's unpaired *t* test using StatView for Windows, version 5.0 (SAS Institute, Cary, NC).

Results

Induction of Id expression by VEGF and TGFβ in HUVECs

To gain insight into the significance of Id in relation to physiological endothelial cell activation and angiogenesis, we examined whether mRNA expression of Id1 and Id3 could be induced by known stimulators of endothelial cells such as VEGF, TGFβ, TNF-α, IL-1β, and bFGF. Significant up-regulation was observed for Id1 and Id3 mRNA when stimulated with VEGF (20 ng/ml) or TGFβ (0.5 ng/ml) (Fig. 1*a*). In contrast, expression of Id was not induced by IL-1β, TNF-α, or bFGF (Fig. 1, *b* and *c*).

Induction of proliferation of HUVECs by overexpression of Id

Next, we asked whether overexpression of Id alone could induce activation and proliferation of HUVECs. To address this question,

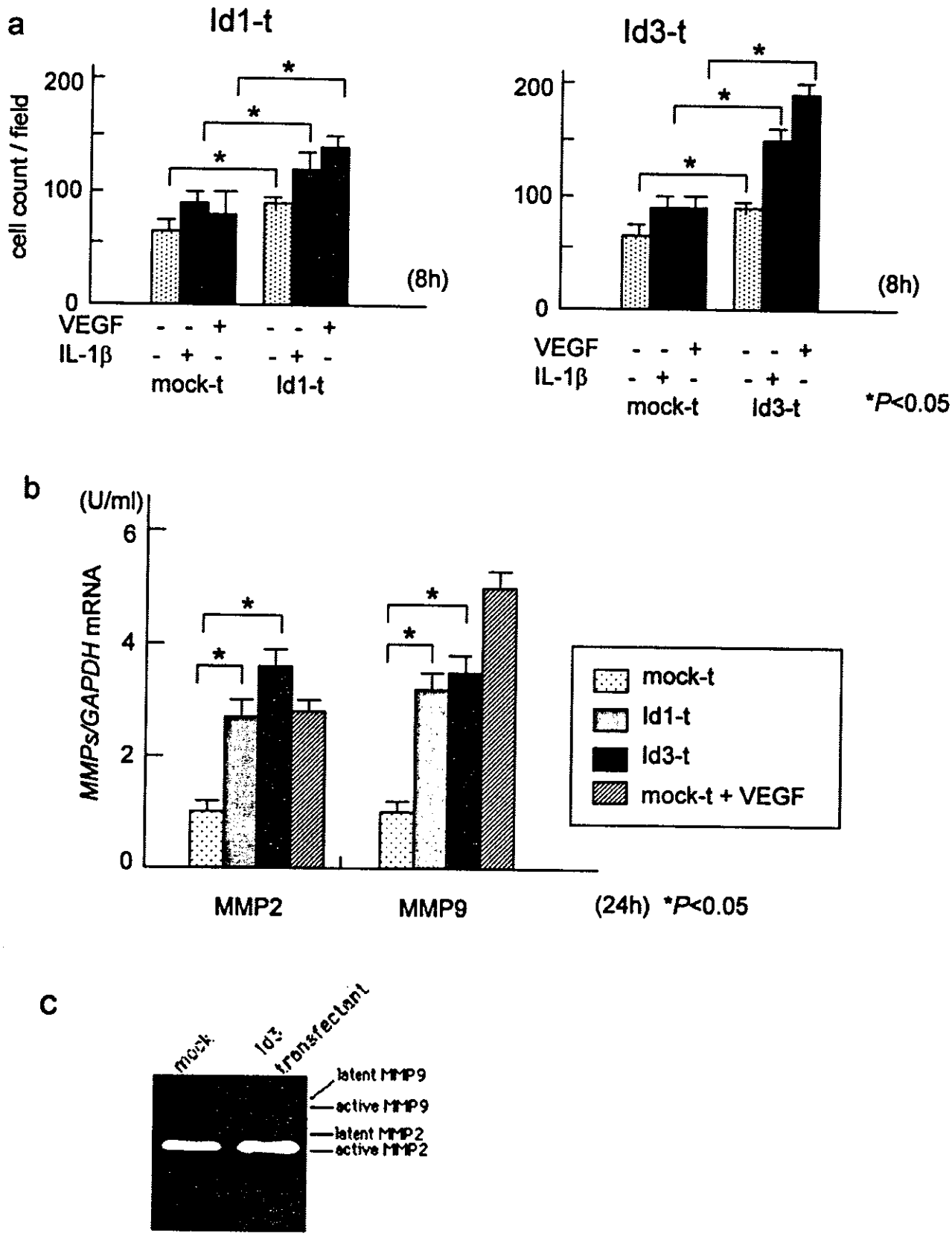

FIGURE 4. Overexpression of Id1 or Id3 induces angiogenic properties in HUVECs. *a*, Transmigration assay. Id1-t and Id3-t demonstrated significant increase in the transmigration activity, especially when the cells were stimulated with IL-1β (0.5 ng/ml) or VEGF (20 ng/ml). *b*, mRNA levels of MMP2 and MMP9. MMP2 and MMP9 expression was significantly increased in Id1-t and Id3-t. *c*, Zymography. Up-regulation of MMP2 and MMP9 activity was confirmed at the protein level.

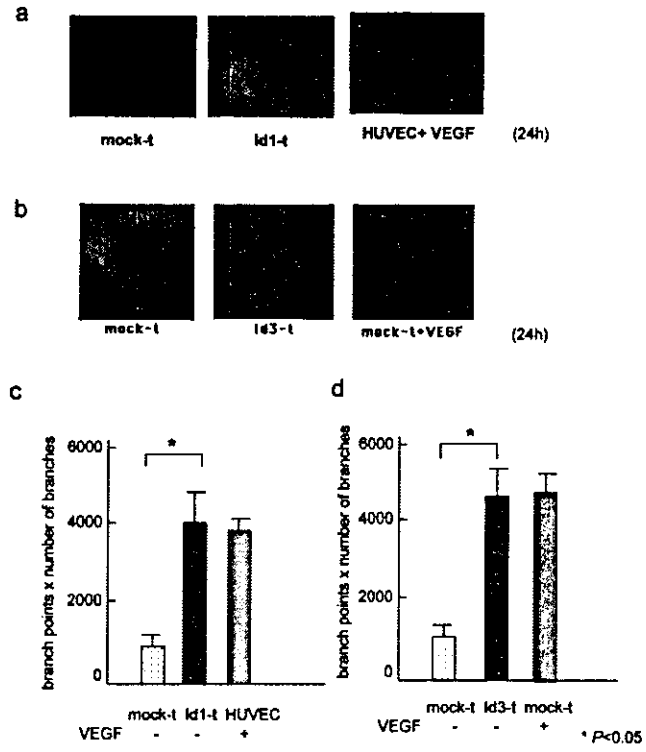

FIGURE 5. Enhanced tube formation by overexpression of Id1 or Id3 in the Matrigel. *a* and *b*, Id1-t (*a*) and Id3-t (*b*) exhibited enhanced tube formation in the Matrigel in the absence of VEGF (original magnification, ×100). *c* and *d*, The degree of tube formation in the transfectants at the basal level was comparable with mock transfectants cultured in the presence of VEGF (10 ng/ml).

transient overexpression of Id1 or Id3 was induced in HUVECs by transfection (Fig. 2*a*). Proliferation was significantly enhanced in both transfectants as compared with mock transfectant, to levels similar to the proliferative activity of untransfected HUVECs when stimulated with VEGF (Fig. 2*b*).

Id has been shown to regulate the cell cycle via transcriptional regulation of $p16^{INK4a}$ (16). In Id transfectants, the $p16^{INK4a}$ expression was found to be inversely correlated with expression level of *ID* mRNA (Fig. 2, *a* and *c*).

Id has also been shown to regulate apoptosis either positively or negatively, depending on the cell types (24, 25). The effect of Id overexpression on apoptosis was examined by serum starvation-induced apoptosis model. After the cells were cultured for 48 h without FBS, Id1 and mock transfectants exhibited comparable enhancement of annexin V binding compared with freshly split HUVEC. In contrast, annexin V binding was significantly inhibited in Id3 transfectants, suggesting that overexpression of Id3, but not Id1, inhibits HUVEC apoptosis induced by serum starvation (Fig. 2*d*).

Induction of activation markers in HUVECs by overexpression of Id

To examine whether Id overexpression alone can induce activation of HUVECs, we measured the expression levels of ICAM-1 (CD54) and E-selectin (CD62E) after transfection of Id1 or Id3 in the absence of VEGF. mRNA levels of ICAM-1 was significantly increased both in Id1 and Id3 transfectants (Fig. 3, *a* and *c*). Correspondingly, the cell surface expression of ICAM-1 was significantly up-regulated in Id1 transfectants (Fig. 3*a*) and, to a lesser extent, in Id3 transfectants (*c*). In contrast, mRNA level and surface expression of E-selectin was clearly up-regulated in Id3 transfectants (Fig. 3*d*) but not in Id1 transfectants (*b*). These results suggested that both Id1 and Id3 can induce activation of HUVEC, but their downstream pathways may not be identical.

Induction of angiogenic processes by overexpression of Id

We next examined whether overexpression of Id in HUVECs can induce angiogenesis. Both Id1 and Id3 transfectants exhibited significant increase in the transmigration activity, especially when the cells were cultured with IL-1β (0.5 ng/ml) or VEGF (20 ng/ml) (Fig. 4*a*).

MMP2 and MMP9 are MMPs relevant to angiogenic processes. Basal expression levels of MMP2 and MMP9 mRNA were significantly increased both in Id1 and Id3 transfectants, and were comparable to those of VEGF-stimulated mock transfectants (Fig. 4*b*). Zymography confirmed up-regulation of MMP2 and MMP9 enzymatic activities (Fig. 4*c*). In the transfectants, not only the total MMP2 and MMP9 expression levels, but also expression levels of the active forms of MMP2 and MMP9 were increased compared with mock transfectants. Furthermore, tube formation in the Matrigel assay was markedly enhanced both in Id1 and Id3 transfectants even in the absence of VEGF (Fig. 5). Taken together, these observations indicate that overexpression of Id alone can induce angiogenic processes in HUVECs.

Inhibition of VEGF-induced proliferation and activation of HUVECs by ID1 *and* ID3 *shRNA*

In the next sets of experiments, we addressed the question whether Id proteins are essential for proliferation, activation, and angiogenic processes of HUVECs induced by VEGF. To inhibit expression of Id1 and Id3, we used RNA interference (RNAi). HUVECs were doubly transfected with RNA expression vectors containing *ID1* and *ID3* shRNA at 0.125, 0.5, or 1 ng/ml. A dose-dependent inhibition of VEGF-induced Id3 (Fig. 6*a*) and Id1 (data not shown) expression was observed. Subsequent studies were conducted using 1 ng/ml *ID1* and *ID3* shRNA transfectants.

Id1/Id3 double-knockdown transfectants exhibited significantly decreased proliferation when stimulated with VEGF (Fig. 6*b*). With respect to adhesion molecules, mock transfectants showed modest up-regulation of surface expression of ICAM-1 (Fig. 6*c*), E-selectin (*d*), and substantial up-regulation of α_v integrin (*e*), when stimulated with VEGF. In *ID1* and *ID3* shRNA transfectants, up-regulation of these molecules were completely inhibited (Fig. 6, *c–e*). When HUVECs were treated with either *ID1* or *ID3* shRNA, VEGF-induced ICAM-1 expression was inhibited, but induction of E-selectin and α_v integrin were not (data not shown).

We next tested whether Id1/Id3 knockdown also inhibits HUVEC activation by IL-1β and TNF-α. IL-1β modestly up-regulated ICAM-1 expression, which was not inhibited by knockdown of Id1 and Id3 (Fig. 6*f*). In contrast, TNF-α-induced up-regulation of E-selectin was inhibited by knockdown of Id1 and Id3 (Fig. 6*g*).

Inhibition of VEGF-induced angiogenesis by ID1 *and* ID3 *shRNA*

We next examined whether Id1/Id3 double knockdown can inhibit the VEGF-induced angiogenic processes of HUVECs. VEGF-induced transmigration activities were abolished in *ID1* and *ID3* shRNA transfectants (Fig. 7*a*). mRNA expression of MMP2, but not MMP9, was significantly decreased by Id1/Id3 knockdown, when HUVECs were cultured in the presence of VEGF (20 ng/ml) (Fig. 7*b*). VEGF-induced tube formation in Matrigel was markedly decreased in *ID1* and *ID3* shRNA transfected cells (Fig. 7, *c* and *d*). Such inhibition of the angiogenic processes was accompanied by a down-regulation of α_2 and β_1 integrins in *ID1* and *ID3* shRNA transfectants (Fig. 6, *h* and *i*). Thus, these results indicate

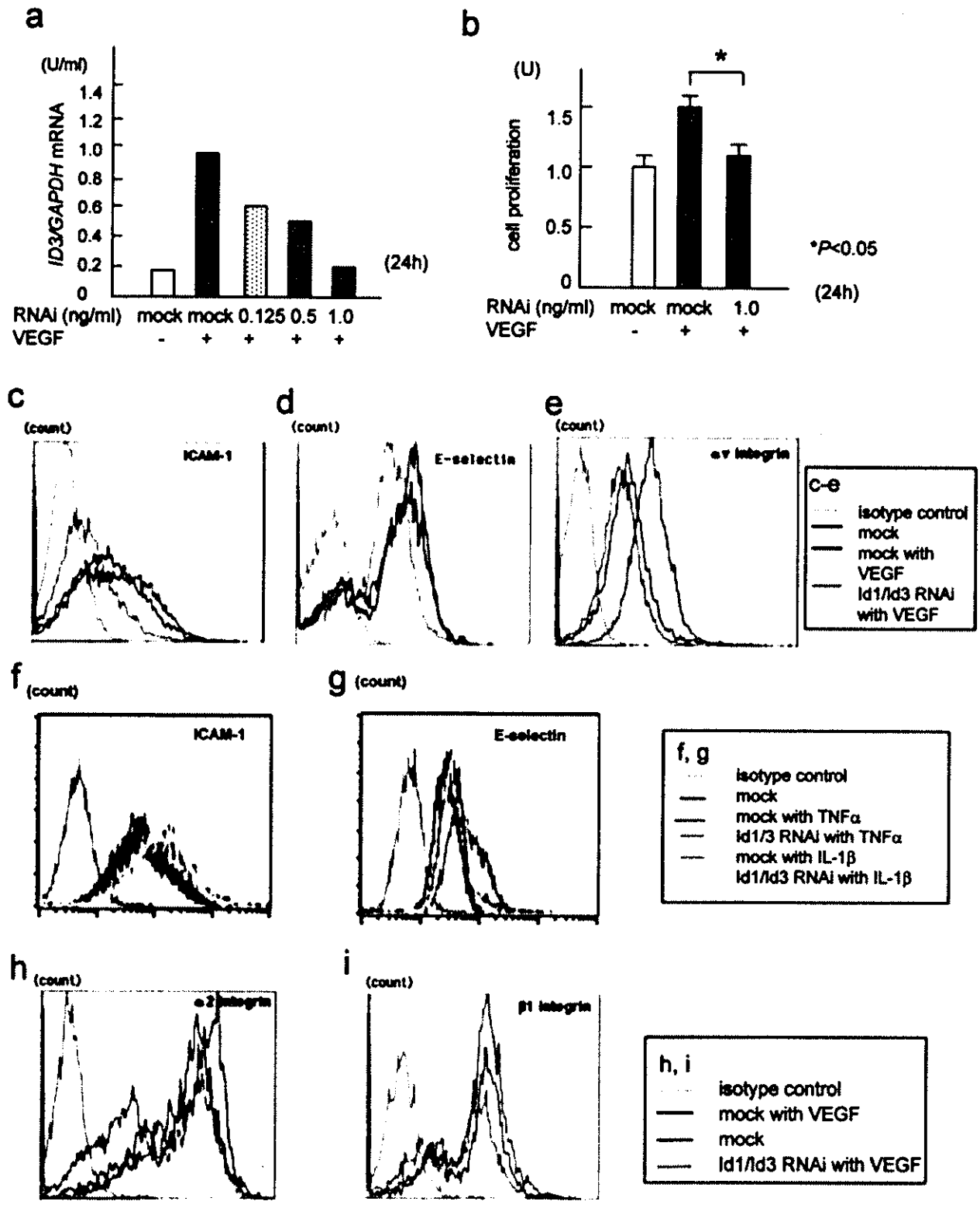

FIGURE 6. Silencing of Id1 and Id3 inhibits VEGF-induced proliferation and activation of HUVECs. *a*, HUVECs were transfected with *ID1* and *ID3* shRNA expression vectors at various concentrations or pSilencer alone (mock). After HUVECs were cultured for 24 h with or without VEGF (20 ng/ml), mRNA levels of *ID3* and *GAPDH* were quantitated. Dose-dependent suppression of *ID3* induction was observed. *ID1* induction was also inhibited (data not shown). *b*, Reduced VEGF-induced proliferation of double-knockdown transfectants. Proliferation was analyzed using WST-1 at 24 h. VEGF-induced proliferation was significantly inhibited in Id1/Id3 double-knockdown transfectants. *c–e*, Inhibition of VEGF-induced activation of HUVECs by Id1/Id3 double knockdown. Surface expression of ICAM-1 (*c*), E-selectin (*d*), and α_v integrin (*e*) was measured in mock transfectants with or without stimulation with VEGF (20 ng/ml), and *ID1* and *ID3* shRNA double transfectants stimulated with VEGF. Id1/Id3 double knockdown resulted in inhibition of VEGF-induced expression of ICAM-1, E-selectin, and α_v integrin. *f*, ICAM-1 expression was modestly up-regulated at 24 h after IL-1β treatment (1 ng/ml) in mock-t, which was not inhibited by knockdown of Id1/Id3. *g*, E-selectin expression was up-regulated at 5 h after TNF-α treatment (20 ng/ml) in mock-t, which was inhibited by silencing of Id1 and Id3. *h* and *i*, Surface expression of α_2 (*h*) and β_1 (*i*) integrins in *ID1*, *ID3* shRNA transfectants stimulated with VEGF (20 ng/ml). Both integrins were down-regulated.

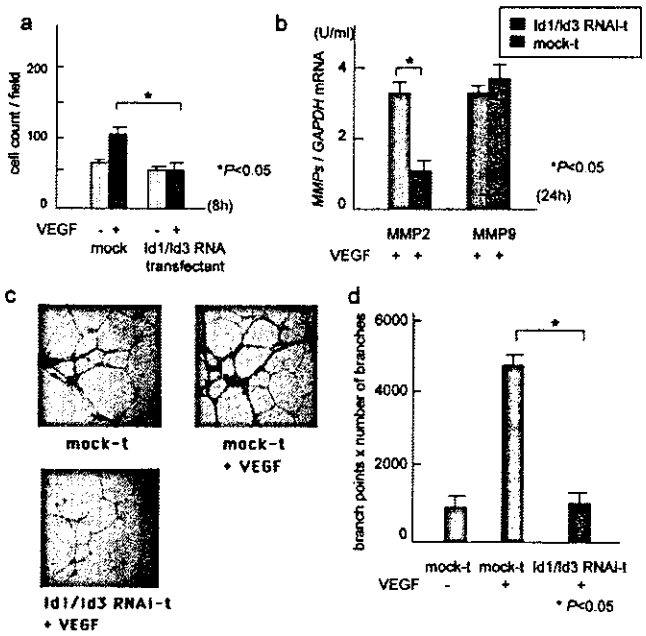

FIGURE 7. Silencing of Id1 and Id3 inhibits VEGF-induced angiogenic processes of HUVECs. *a*, Transmigration activity was significantly decreased in *ID1* and *ID3* shRNA transfectant after stimulation with VEGF (20 ng/ml) for 8 h. *b*, MMP mRNA expression in mock or *ID1* and *ID3* shRNA transfectants cultured for 8 h in the presence of VEGF (20 ng/ml). MMP2 mRNA expression was decreased in the double-knockdown cells, whereas MMP9 mRNA was not inhibited. *c*, Tube formation assay. Untransfected, mock-transfected, and *ID1*, *ID3* shRNA double-transfected HUVECs were cultured in the presence of VEGF (20 ng/ml) for 24 h. Marked inhibition of tube formation was observed by Id1/Id3 double knockdown. Original magnification, ×100. *d*, Quantitation of tube formation.

that the expression of Id plays a crucial role in the VEGF-induced angiogenic processes in HUVECs.

Discussion

In the present study, we demonstrated that overexpression of Id alone can induce proliferation, activation, and angiogenic processes of HUVECs in the absence of VEGF, to levels similar to that of VEGF-stimulated untransfected HUVECs. Moreover, knockdown of *ID1* and *ID3* in HUVECs almost completely abolished the VEGF-induced proliferation, activation, and angiogenic processes. These findings indicate a crucial role for Id in some of the VEGF signaling pathways in HUVECs.

Using $Id1^{+/-}Id3^{-/-}$ mice, it has been shown that Id expression is required to support angiogenesis in tumors (19), and that recruitment of $VEGFR1^{+}$ myeloid cells and $VEGFR2^{+}$ circulating endothelial precursor cells expressing Id is necessary for tumor growth (26). The new information presented in this study, including the requirement of Id also in human endothelial cells, up-regulation of ICAM-1 and E-selectin by forced expression of Id, and inhibition of endothelial cell activation and angiogenesis by double knockdown of Id1 and Id3, further emphasizes the crucial role of Id in endothelial cell activation and angiogenesis.

Previous reports demonstrated that Id controls the cell cycle in several ways (15), for example, by binding to Rb protein and blocking its tumor suppressor function (6, 12), or by inhibiting the binding of Ets1 and Ets2 transcription factors to $p16^{INK4a}$ promoter and repressing its expression (16). Our data suggested that at least the latter mechanism is operative in HUVECs. Id family proteins have been generally shown to promote apoptosis in a variety of conditions (24). However, in some settings, Id was shown to have antiapoptotic activity (25). Our present data suggested that overexpression of Id3, but not Id1, can protect HUVEC from apoptosis induced by serum starvation. In HUVECs, it was previously shown that VEGF prevents apoptosis induced by serum starvation (22). These results suggest that Id3 may mediate antiapoptotic effect induced by VEGF in HUVECs, and such an effect may also partly account for enhanced proliferation of Id3 transfectants.

As for ICAM-1 and E-selectin induction, it was recently demonstrated that VEGF induces these adhesion molecules in HUVECs through NF-κB (27), and that Id1 activates NF-κB transcription in prostate cancer cells (25). Our present data provide evidence that Id is involved in the signaling pathway that connects VEGF receptors and NF-κB activation in HUVECs. In our system, we have not distinguished contribution of each VEGFR for the induction of Id, which should be investigated in future studies. Although gene silencing of Id1 and Id3 efficiently inhibited ICAM-1 and E-selectin induction by VEGF and TNF-α, up-regulation of ICAM-1 by IL-1β was not inhibited. Taken together with the lack of *ID1* and *ID3* mRNA induction after stimulation with IL-1β, our results suggest that Id may not be involved in the IL-1β pathway of HUVEC activation. In the case of TNF-α stimulation, because mRNA of *ID* was not up-regulated, the presence of basal level of Id may be necessary for the activation of HUVEC by TNF-α. The signaling pathways of VEGF, TNF-α, and IL-1β in endothelial cells have not yet been fully delineated (28), and further studies are necessary to address the relationships of these pathways and Id.

When HUVECs were treated with VEGF or TGFβ, expression of Id was induced. Expression of Id3 has been shown to be induced by the Ras-ERK pathway in thymocytes (17), and through type I receptor of the TGFβ via Smad1/5 signaling in HUVECs (18), whereas Id1 expression was associated with Raf/MEK1/2 activation in a human prostatic cancer cell line (29) and Smad1/5 signaling in HUVECs (18). Induction of Id in HUVECs by VEGF is reasonable, because MAPK cascade exists in the downstream of VEGF signaling pathways (30). In contrast, stimulation with IL-1β, TNF-α, or bFGF did not induce Id1 or Id3 in HUVECs, suggesting that Id may not be a ubiquitous mediator of inflammation, but more specifically associated with signaling pathways leading to angiogenesis.

For the knockdown experiments, we used double knockdown of Id1 and Id3, because knockdown of either one of them resulted in only partial inhibition of VEGF-induced activation or angiogenesis. Such results were expected, because it has been demonstrated that Id1 and Id3 have similar promoter region sequences, and exert overlapping biochemical functions; thus either one might be able to compensate the other, at least partially. In contrast, our results indicated that knockdown of both Id1 and Id3 was sufficient to abolish most of the angiogenic processes induced by VEGF in HUVECs, and Id2 and Id4 cannot compensate for the lack of Id1 and Id3. *ID1* and *ID3* shRNA sequences were designed from the 5′ region specific to each gene. Because RNAi can block gene expression only when the shRNA sequence is highly matched with the target gene (31), Id2 and Id4 expression were not considered to be suppressed.

Although Id3 overexpression up-regulated MMP9, knockdown of Id did not inhibit VEGF-induced up-regulation of MMP9. This suggests the presence of other pathways that lead to MMP9 up-regulation. Nevertheless, Id knockdown resulted in marked inhibition of tube formation in Matrigel. Such discrepancy may be explained by reduced expression of α_2 and β_1 integrins, whose

ligands are collagen and laminin, the main components of basement membranes and also of Matrigel (32, 33). Thus, Id seems to be involved in multiple pathways leading to activation of endothelial cells and angiogenesis. Although the functions of Id1 and Id3 are substantially overlapping (11, 14), it is intriguing to delineate the pathways from each Id protein to a multitude of endothelial cell activation and angiogenic processes. Indeed, our observations indicated some notable differences between the effects of Id1 and Id3 overexpression; for example, Id1 overexpression failed to up-regulate E-selectin and to protect HUVEC from apoptosis induced by serum starvation. It has recently been reported that Id1 down-regulates expression of thrombospondin-1, a suppressor of angiogenesis (34). It is also of interest to examine whether all of the angiogenic processes associated with Id can be explained by down-regulation of thrombospondin-1. To gain further insights, DNA microarray analysis is underway.

In conclusion, we demonstrated that Id plays an indispensable role for the VEGF-induced activation and angiogenic processes of HUVECs. Based on the inhibition of endothelial cell activation and angiogenesis with RNAi, as well as the low expression of Id in most of the normal adult tissues, Id should be considered an attractive target for the development of new therapeutic approaches for disorders associated with excessive angiogenesis.

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Follistatin-related protein gene (*FRP*) is expressed in the synovial tissues of rheumatoid arthritis, but its polymorphisms are not associated with genetic susceptibility

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Abstract

Objective

To examine the expression level and function of follistatin-related protein gene (FRP, also referred to as FSTL1) in rheumatoid arthritis (RA), and possible association of its polymorphisms with genetic susceptibility to RA.

Methods

FRP mRNA expression levels in the synovial tissues from 10 patients with RA and 5 patients with OA were measured using real-time RT-PCR. Effects on the growth of synovial cells were evaluated by stably introducing FRP cDNA into a rheumatoid synovial cell line, E11. Screening of genomic DNA variations was done using DNA from 12 patients with RA and 12 healthy individuals by direct sequencing. Genotypes at the detected polymorphic sites were determined in 224 patients with RA and 220 healthy individuals using PCR-single strand conformation polymorphism.

Results

FRP mRNA was overexpressed in synovial tissues from RA patients by 2.3-fold as compared with those from OA. A rheumatoid synovial cell line (E11) transfected with FRP exhibited reduced proliferation, probably mediated by secreted FRP molecule. 16 genomic variations were identified, among which 4 were polymorphisms within the promoter region and exons, and the remainder were either rare variations or intronic polymorphisms. Genotyping of 4 polymorphic sites did not reveal statistically significant association with the susceptibility to RA.

Conclusion

FRP mRNA is overexpressed in RA synovium, the product of which exerts inhibitory activity on synovial cell growth. Although new polymorphic sites were identified, they were not associated with susceptibility to RA, suggesting that overexpression of FRP is secondarily caused by synovial environment of RA.

Key words

Rheumatoid arthritis, synovium, gene expression, polymorphism, genetics.

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Introduction

Rheumatoid arthritis (RA) is characterized by abnormal proliferation of synovium and chronic inflammation in joints throughout the body that eventually result in bone and cartilage resorption. It is thought that multiple genetic factors take part in the onset of RA; however, disease-susceptibility genes other than HLA-DRB1 have not been established (1, 2).

One of the efficient approaches to find genes relevant to the pathogenesis or etiology of RA is the use of comprehensive mRNA expression profiling. Genes significantly upregulated or downregulated in the synovial tissues of RA are likely to have a functional role, and polymorphisms in the coding or regulatory sequences of such genes might be associated with susceptibility to RA. We recently analyzed mRNA expression profiles using differential display, and found some genes substantially upregulated in a synovial tissue from a patient with RA compared with that from a patient with osteoarthritis (OA), one of which was a gene coding for follistatin-related protein (*FRP*, also referred to as follistatin-like 1 [*FSTL1*]) (3).

FRP is a putative secretory protein with a molecular weight of 50-55kDa, composed of 308 amino acids. *FRP* gene was originally described as TGF-β-stimulated clone (TSC)-36, which was induced when transforming growth factor (TGF)-β1 was added to mouse osteoblast cell line MC3T3-E1 (4). It is known that the product of mouse TSC-36 gene has high homology (> 92%) with the amino acid sequence of human FRP; therefore human *FRP* is likely to be a homolog of mouse TSC-36 in terms of function (5). In addition, it is reported that the amino acid sequence of TSC-36 is similar to that of follistatin, and that transcription factor AP (activator protein)-1 contributes to the expression of *FRP* gene (6).

It was recently reported that 44% of patients with RA possess autoantibody to FRP (7). Subsequently, it was demonstrated that FRP is induced by TGF-β in fibroblast-like synoviocytes, and that FRP downregulates production of matrix metalloproteinase (MMP)-1, MMP-3 and prostaglandin E_2 (PGE_2) in the synovial cell lines (8). Based on these observations, it has been proposed that autoantibody to FRP blocks such potentially protective activity of FRP against joint destruction (8).

Interestingly, the chromosomal position of *FRP* gene (3q13.33) coincides with one of the candidate loci reported from genome-wide linkage studies (9, 10), suggesting that polymorphism of *FRP* might be associated with susceptibility to RA.

In the present study, we made an attempt to examine the mRNA expression level of *FRP* in multiple patients with RA and osteoarthritis (OA), and to gain insight into its functional role by introducing *FRP* gene into a synovial cell line. We also screened for polymorphisms of human *FRP* gene, and examined whether they are associated with the susceptibility to RA.

Materials and methods

Patients and healthy individuals

Synovial tissues were obtained from 10 patients with RA as well as from 5 patients with OA, undergoing total knee replacement surgery for a therapeutic purpose. The patients are among those whose clinical characteristics were previously described (3).

Genomic DNA was obtained from 224 patients with RA (male 28, female 196, 45.2 ± 13.5 years) and 220 healthy individuals (male 131, female 89, 33.3 ± 9.6 years). RA was diagnosed according to the American College of Rheumatology criteria (11). All individuals were genetically unrelated Japanese living in Tokyo area. This study was reviewed and approved by the Research Ethics Committee of the Graduate School of Medicine, the University of Tokyo.

Transfection and proliferation assay

A rheumatoid synovial cell line, E11 (12,13), was maintained in DMEM medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), antibiotic/antimycotic solution (Sigma-Aldrich) and 2 mM L-glutamine. Full length cDNA of *FRP* was prepared by RT-PCR using primers 5'-ACCAGACCACGATGTG-

GAA-3' (forward) and 5'-GATCTC-TTTGGTGCTCACTCTC-3' (reverse), and was stably introduced to E11 using pcDNA 3.1/V5-His vector (Invitrogen, Carlsbad, CA), and Effectene Transfection Reagent (Qiagen).

The efficiency of transfection to E11 cells was estimated as follows. 0.5 x 10^6 of E11 were seeded 24 h before pSV-β-galactosidase Control Vector (Promega, Madison, WI) was introduced. After 24 h, the cells were stained with X-Gal Staining Assay Kit (Gene Therapy Systems, San Diego, USA). The transfection efficiency was estimated to be 38.6 ± 2.6% from three independent experiments.

Proliferation of E11-mock and E11-*FRP* transfectants were assayed using WST-1 cell proliferation assay (Takara Bio, Otsu, Japan), in accordance with the manufacturer's instructions.

The inhibitory activity of the culture supernatant of E11-*FRP* transfectants was examined by transfer experiments. Culture supernatant from either E11-mock or E11-*FRP* transfectants was collected after culture for 24 h, and E11 cells were cultured in each of the supernatants. Proliferation after 24 h and 48 h was measured using WST-1 assay.

RNA preparation

Synovial tissues were obtained from surgically removed knee joints, frozen with liquid nitrogen, and kept at –80°C until use. Frozen tissues were crashed and subjected to total RNA extraction using TRIZOL reagent (Life Technologies, Rockville, MD). RNA was prepared from the cultured cells using RNeasy (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Real-time RT-PCR

To compare mRNA levels of *FRP* between RA (n=10) and OA synovia (n=5), and between E11-*FRP* transfectant and mock transfectant, real time RT-PCR was performed with LightCycler (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The expression of *FRP* mRNA was standardized using that of β-actin mRNA. Primer sets used are the following: *FRP*: 5'-CATTCCA-GATGGCTGGTTCT-3' (forward), 5'-TGCATACGTTTCATCCTCCA-3' (reverse); β-actin: 5'-TCCTGTG-GCATCCACGAAACT-3' (forward), 5'-GAAGCATTTGCGGTGGACGAT-3' (reverse).

ELISA

TGF-β concentration in the culture supernatant was measured by ELISA using Quantikine Human TGF-β1 Immunoassay (R&D, Minneapolis, MN) at 24 h and 72 h after 4 x 10^5 cells of *FRP*- and mock-transfected E11 were seeded into 6-well culture plates.

Variation screening and genotyping of genomic DNA

Genomic DNA for variation screening and genotyping were purified from peripheral blood leukocytes using a QIAamp blood kit (Qiagen).

Genomic configuration of human *FRP* gene was elucidated using genomic DNA sequence (GenBank accession No. NT_005612) and mRNA sequence (NM_007085) in combination. Variation screening of the promoter region (up to -1.6 kb) and all exons was performed on genomic DNA from 12 patients with RA and 12 healthy individuals by direct sequencing. Direct sequencing was performed by an automated sequencer (Applied Biosystems, Foster City, CA) using dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Genotyping and association studies were performed for the detected polymorphic sites in the promoter region and in the exons using PCR–single strand conformation polymorphism (SSCP), as previously described (14).

The primers and experimental conditions used for genotyping are shown in Table I. Those used for variation screening are available upon request.

Statistical analysis

The differences in the mRNA levels between RA and OA synovia were statistically analyzed using Mann-Whitney's U test, using StatView-J4.11 for Macintosh (Abacus Concepts Inc., Berkeley, CA).

The genotype frequencies of the detected polymorphisms were compared between patients with RA and healthy individuals using the χ^2 test and Fisher's exact test. Fisher's exact test was used when one or more cells in 2 x 2

Table I. Primers and experimental conditions used for genotyping.

Primer	Position	Sequence (5' → 3')	AT (°C)	Size (bp)	Typed SNP	AA (%)	Temp (°C)	Time (min)	Gly
FRP-PRO8L	promoter	CCCTGTCAAAGAGGTAGCACA	65.2	275	-919C>G, -832G>A	12.5	10	120	+
FRP-PRO8R		TGCTGTGGGTTCTACAGTGC							
FRP-PRO14L	promoter	ACAACAGTGGGCACTCAACA	64.9	282	-1572T>A	10	20	120	-
FRP-PRO14R		CCACTGCAATCCATTGTCATA							
FRP-EX11-SSCPL	exon 11	TGCATCACGATTGAAAGAGG	63.5	301	c*.414T>C	12.5	20	120	-
FRP-EX11-SSCPR		TCCCAGAAACTCCATCCAAG							

AT: annealing temperature; Size: size of PCR products; AA: concentration of acrylamide in SSCP gel; Temp: Electrophoresis temperature; Time: duration of electrophoresis, Gly: presence or absence of 10% glycerol in SSCP gel.

contingency tables contained a value of less than 5. A P value < 0.05 was regarded as statistically significant.

Linkage disequilibrium (LD) analysis and estimation of haplotype frequencies were performed by the Estimation-Maximization (EM) algorithm using SNPalyze program (DYNACOM, Mobara, Japan). The extent of LD was assessed by Lewontin's D' and P values (15).

Results

Overexpression of FRP in synovial tissues of RA

To examine whether the previously reported upregulation of *FRP* in a synovial tissue from a patient with RA detected in differential display (3) is commonly observed in the majority of patients with RA, a quantitative real-time RT-PCR was performed and mRNA levels were compared between the synovial tissues from 10 patients with RA and 5 patients with OA. As shown in Figure 1, mean mRNA level of *FRP* was elevated in RA by 2.3-fold (P = 0.01), indicating that the upregulation of *FRP* is generally observed in the synovial tissues from RA.

Inhibitory activity of FRP on the growth of a rheumatoid synovial cell line E11

Next we examined for potential effects

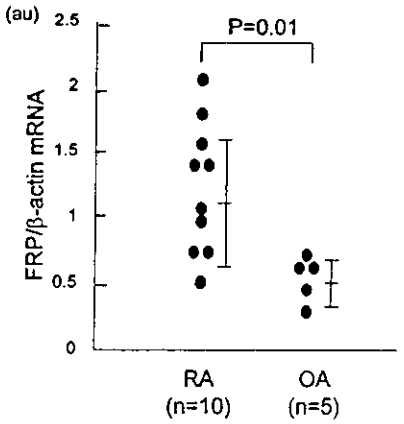

Fig. 1. mRNA levels of *FRP* in the synovia from RA as compared with OA. The mRNA concentrations of *FRP* were quantitated using real-time RT-PCR, standardized by the concentration of β-actin mRNA, and the results were expressed as arbitrary units. *FRP* mRNA level in RA synovia was 2.3-fold upregulated compared with those in OA synovia (P = 0.01, Mann-Whitney's U test).

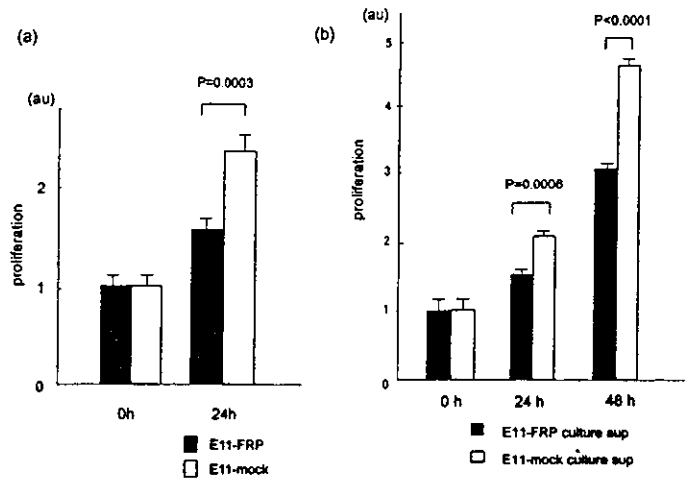

Fig. 2. (a) Inhibition of proliferation of a rheumatoid synovial cell line, E11, by transfection of *FRP*. E11 stably transfected with *FRP* and mock transfectants were cultured for 24 h and the cell numbers were measured using WST-1 proliferation assay. Results are shown in arbitrary units (au) calculated from absorbance. E11-*FRP* demonstrated significantly decreased proliferation as compared with mock transfectants. (b) Growth inhibitory activity of E11-*FRP* culture supernatants. Culture supernatant from either E11-mock or E11-*FRP* transfectants was collected after culture for 24 h, and E11 cells were cultured in each of the supernatants. Proliferation after 24 h and 48 h was measured using WST-1 assay. Significant inhibition was observed when the cells were cultured in the supernatant from E11-*FRP*.

of FRP on the growth of a rheumatoid synovial cell line, E11, by stably introducing *FRP* cDNA. The expression level of FRP in the tranfectants was found to be twice as high as that of the mock transfectants in three independent experiments. As shown in Figure 2a, E11-*FRP* transfectants demonstrated significantly decreased proliferation compared with mock transfected E11 (P = 0.0003).

To test whether this inhibitory effect was mediated by secreted or membrane-bound factors, proliferation was compared between E11 cells cultured in the supernatants from E11-mock and from E11-*FRP*. As shown in Figure 2b, proliferation was significantly decreased in the E11 cells cultured in the supernatants from E11-*FRP*, indicating that soluble factor(s), possibly *FRP* itself, is responsible for the inhibitory activity of E11-*FRP*.

To exclude the possibility that TGF-β was responsible for the growth inhibitory effect of the *FRP*-transfectants, TGF-β1 was measured in the culture supernatants of the *FRP*- and mock-transfectants. The concentration of TGF-β1 in *FRP* transfectants was 0.14 (24 h) and 1.30 ng/ml (72 h), which was lower than that in mock transfectants (0.22 and 1.87 ng/ml, respectively).

Variation screening and association study

Genomic DNA variation screening revealed 4 variations in the promoter region (-1572T>A, -1427C>T, -1390G>A and -919C>G), 4 in exons (-229C>A, c.716C>T [Thr239Met], c*.411A>G and c*.414T>C) and 7 in introns (IVS1+47G>T, IVS1+103G>A, IVS1+109_111delGGG, IVS2-27G>T, IVS3+50_51insC, IVS3-73_-72insGT, IVS3-69G>A) (The sequence variations were described according to the nomenclature system proposed by den Dunnen [16]) (Fig. 3). In addition, in the course of genotyping, another variation in promoter region (-832G>A) was detected.

A case-control association study was performed for 4 variations that were located in the promoter region or exons and at the same time met the definition of polymorphism (major allele frequency < 99%). Statistically significant association was not detected for any of these four single nucleotide polymorphisms (SNPs) (Table II). Others were either rare variations or intronic variations.

Haplotype frequencies and pairwise linkage disequilibrium (LD) between SNPs are demonstrated in Table III. Strong LD was observed between promoter region SNPs, while LD between

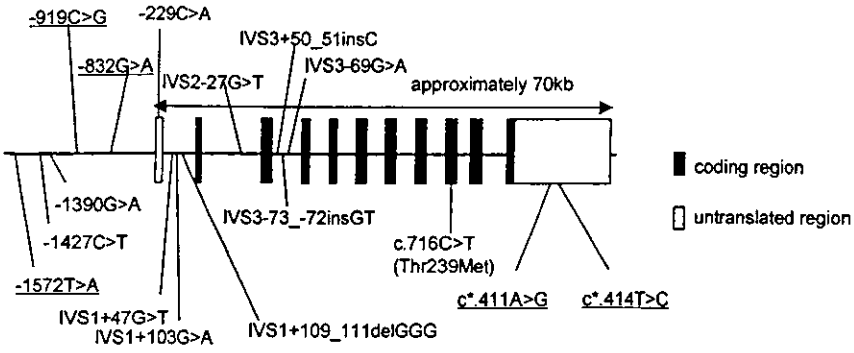

Fig. 3. Structure of the human *FRP* gene and variations detected in this study. Underlined are the SNPs within the promoter region and exons genotyped in this study. Others are either rare variations (allele frequency < 1%) or polymorphisms within introns. The designation of the variations was based on (16). The nucleotide sequences of these variations will appear in the DDBJ/EMBL/GenBank database under the accession number AB119283.

3' UTR SNP and promoter SNPs was modest. Haplotype frequencies of RA patients did not significantly differ from these of healthy controls.

Discussion

In the present study, we confirmed overexpression of *FRP* mRNA in the synovial tissues of patients with RA as compared with OA. It was previously shown in a small-scale study that *FRP* mRNA was increased in the synovial tissues of RA as compared with OA, while the concentration of *FRP* protein was not significantly different between synovial fluids from patients with RA and OA, irrespective of the presence of anti-FRP autoantibodies (7). Such a discrepancy between mRNA and protein levels might be attributable to the accelerated turnover of *FRP* in RA, as has been suggested (7).

The source of *FRP* in the synovial tissues remains unclear. A previous study demonstrated that *FRP* is rather ubiquitously expressed except for peripheral blood leukocytes (7); however, the possibility that the difference between RA and OA could be due to differences in the number of infiltrating inflammatory cells cannot be excluded. To address this issue, *FRP* mRNA levels were compared in fibroblast-like synoviocytes from each two patients with RA and OA; however, the number of the samples was too small to make any meaningful interpretation possible (data not shown). This question needs to be addressed in the future, for instance by immunohistochemistry techniques, when antibody to FRP becomes available. Since FRP is considered to be a secreted molecule, it should be possible to exert its effect even if it is expressed mainly in the infiltrating inflammatory cells, rather than the synoviocytes.

It was of particular interest to explore the molecular mechanisms responsible for the induction of such overexpression. In this study, we examined the possibility that polymorphisms within the *FRP* gene, especially those in the regulatory region, might be associated with RA. Although we identified a number of new variations, significant association with RA was not detected. There are some limitations in this study, which might possibly have led to false-negative results. The male-to-female ratio was substantially different between RA and controls; however, since the genotype frequencies were not different between male and female controls, the results were essentially identical after adjustment for gender ratio (data not shown). Although the age distribution of the controls was younger than the patients, potential misclassification caused by future development of RA among controls is less than 1% (the prevalence of RA in Japan); therefore, it is unlikely that such age difference significantly affected the results. The variations were screened in all exons, intronic regions flanking exons and the promoter region up to -1.6 kb, but the possibility that variations in other regions, for example in the middle of introns or in the further upstream promoter region, might be associated with RA cannot theoretically be excluded. In addition, the number of subjects is not sufficiently large to

Table II. Genotype and allele frequencies of SNPs in the promoter and 3'-untranslated regions of *FRP*.

SNP	Genotype, allele	RA (n=224)	(%)	Controls (n=220)	(%)
-1572T>A	T/T	200	(89.3)	197	(89.5)
	T/A	23	(10.3)	21	(9.6)
	A/A	1	(0.4)	2	(0.9)
	T	423	(94.4)	415	(94.3)
	A	25	(5.6)	25	(5.7)
-919C>G	C/C	198	(88.4)	197	(89.6)
	C/G	25	(11.2)	19	(8.6)
	G/G	1	(0.4)	4	(1.8)
	C	421	(94.0)	413	(93.9)
	G	27	(6.0)	27	(6.1)
-832G>A	G/G	215	(96.0)	215	(97.7)
	G/A	9	(4.0)	5	(2.3)
	A/A	0	(0)	0	(0)
	G	439	(98.0)	435	(98.9)
	A	9	(2.0)	5	(1.1)
c*.414T>C	T/T	96	(42.9)	100	(45.5)
	T/C	102	(45.5)	98	(44.5)
	C/C	26	(11.6)	22	(10.0)
	T	294	(65.6)	298	(67.7)
	C	154	(34.4)	142	(32.3)

Significant difference between RA and controls was not observed in any of the SNPs.

Table III. Estimated haplotype frequencies and pairwise linkage disequilibrium between SNPs.

(a) Estimated haplotype frequencies.

Haplotype	-1572	-919	-832	c*.414	Haplotype frequency (%) RA	controls
1	T	C	G	T	59.9	61.5
2	T	C	G	C	31.9	31.2
3	A	G	G	T	3.5	4.7
Others					4.7	2.6

(b) Pairwise linkage disequilibrium between SNPs

SNPs		Controls -919	Controls c*.414	RA -919	RA c*.414
-1572	*D'*	1	0.436	0.957	0.019
	P	0.000	0.177	0.000	0.797
-919	*D'*		0.495		0.054
	P		0.045		0.906

detect an association of rather weak contribution. However, at this point, it is more probable that overexpression of *FRP* is caused by inflammatory synovial environment in RA, rather than genomic polymorphisms of *FRP* gene.

Another interesting finding in this study was the inhibitory activity of FRP on the growth of rheumatoid synovial cell line, E11. Inhibitory effect of TSC-36, the homologue of FRP in mice, on the growth of human lung cancer cell has been reported (17). With respect to human synovial cells, inhibitory activity of FRP has been demonstrated on the production of mediators of joint destruction, such as MMP-1, MMP-3 and prostaglandin E2 (8). A recent study further provided evidence that administration of FRP into mouse joints ameliorates joint destruction (18). Our finding provided further support that FRP may exert a protective effect for joint destruction on synoviocytes. Our transfer experiment suggested that secreted factor(s) other than TGF-β is responsible for the growth inhibitory effect. Owing to the lack of commercial antibodies to FRP, the possibility that secreted FRP itself is the inhibitory factor could not be addressed in this study, and needs to be confirmed in the future. Why joint damage progresses in spite of overexpression of *FRP* remains a mystery. The presence of neutralizing antibody to FRP in patients with RA may provide one explanation (7,8). Another possibility may be that FRP has unknown functions on cells other than synoviocytes, for example osteoclasts, that are agonistic for inflammation and/or joint damage. The mechanism of regulation of FRP as well as its effects on various cell lineages that constitute inflammatory joints requires further study.

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Heat shock protein 90 is required for increased DNA binding activity of activator protein-1, a heterodimer of Fos/JunD, in rheumatoid synovial cells under inflammatory stimuli

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Abstract. We have studied the DNA binding profiles of activator protein-1 (AP-1) involved in synovial overgrowth and osteoporosis in rheumatoid arthritis (RA) in relation to the molecular chaperon heat shock protein 90 (HSP90). The AP-1 binding activity of the nuclear extracts of rheumatoid synovial cells was basically increased as compared with osteoarthritic synovial cells. Upon stimulation with inflammatory cytokines IL-1ß or TNFα, the AP-1 binding activity was further increased in rheumatoid synovial cells, and increased AP-1 protein was composed as heterodimers of Fos and JunD which was not known before as a major component of AP-1 in rheumatoid synovial cells. The increase of AP-1 binding activity as induced by inflammatory cytokines was specifically inhibited by geldanamycin, radicicol or herbimycin A, specific inhibitors of HSP90, while AP-1 proteins was not decreased by geldanamycin. Further, HSP90 protein was not decreased by the inhibitors. The findings indicate that HSP90 is required for increased AP-1 binding activity of rheumatoid synovial cells under inflammatory stimuli and that AP-1 binding activity is inhibited by functionally inactivating HSP90 with the inhibitors.

Introduction

Rheumatoid Arthritis (RA) is a chronic polyarthritis of unknown etiology characterized by progressive joint destruction (1). It has previously been shown that synovial overgrowth and osteoporosis are produced by augmenting c-*fos* gene expression (2-6). Overexpression of c-Fos/activator protein-1 (AP-1) also contributed to dysregulated cell cycle progression: previous studies showed that c-Fos/AP-1 directly transactivated the *wee1* kinase gene at the G1/S phase of the cell cycle, and that transient increase of c-*fos* and *wee1* kinase genes prevented cellular premature mitosis while the cells remained in the G1/S phase of cell cycle (7). However, while c-Fos makes a heterodimer complex with Jun family members to regulate gene expression (8,9), composition of AP-1 heterodimer and the dynamics of AP-1 binding activity remain unclear (10,11).

In the present study, we have characterized AP-1 in relation to molecular chaperon heat shock protein 90 (HSP90). HSP90 is a recently identified molecular chaperon belonging to the HSP family, which is massively present in the cell, and the concentration of which is increased subject to stressors including heat, reactive oxygen, low pH, anoxia and toxins (12). HSP90 is specifically inhibited by benzoquinone ansamycin geldanamycin, an antibiotic that binds specifically with the N-terminal ADP/ATP binding pocket of HSP90 molecule and interferes with its chaperoning action (13,14). HSP90 is also specifically inhibited by macrocyclic antifungal antibiotic radicicol (15), and benzoquinoid ansamycin herbimycin A (16). In particular, geldanamycin has been shown to inhibit tumor progression and, for this reason, is currently under clinical trial as an anti-tumor agent (17).

We found that, upon stimulation with inflammatory cytokines IL-1ß or TNFα, the DNA binding activity of AP-1 was significantly increased in rheumatoid synovial cells, in which Fos and JunD heterodimer was the major constituent of AP-1. The AP-1 binding activity thus increased by inflammatory cytokines was inhibited specifically by the inhibitors of HSP90, indicating that HSP90 is required for the binding of Fos/JunD to AP-1 binding sites in rheumatoid synovial cells under inflammatory stimuli.

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Key words: rheumatoid arthritis, activator protein-1, JunD, heat shock protein 90, geldanamycin

Materials and methods

Cell culture. Samples of synovium were obtained during joint surgery from patients with RA and osteoarthritis (OA), following the world medical association declaration of Helsinki ethical principles for medical research involving human subjects. The diagnosis of RA was made according to the 1987 revised criteria of American College of Rheumatology (18). Tissues were minced and digested in a solution containing 0.2% of collagenase (Sigma, St. Louis, MO) and 0.25% of trypsin-ethylenediamine tetraacetic acid (trypsin-EDTA; Difcol Laboratories, Detroit, MI) at 37°C for 1 h. Dispersed rheumatoid synovial cells were cultured in a 10 cm dish (Asahi Techno Glass Corp., Tokyo, Japan) with DMEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% FCS (Biosciences Pty Ltd., Lome, Australia) supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin. After overnight culture, non-adherent cells were removed, and adherent cells were further incubated in fresh medium. All experiments were conducted using the cells of 3-4 passage.

Preparation of nuclear extracts. Synovial cells ($3x10^5$) were treated with ~100 nM of geldanamycin (Sigma-Aldrich, St. Louis, MO), ~10 nM of radicicol (Sigma-Aldrich) or ~100 nM of herbimycin A (Sigma-Aldrich) for 12 h. The cells were cultured with 4 ng/ml of IL-1ß (Roche Diagnostics, Manheim, Germany) or 20 ng/ml of TNFα R&D systems Inc., Minneapolis, MN) for ~24 h. After washing, they were suspended in 200 µl of hypotonic buffer (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 1 µM leupeptin and 1 µM aprotinin). After adding 2 µl of 10% Nonidet P-40, cells were incubated on ice for 10 min and centrifuged at 2,000 g for 30 sec, and the fraction containing nuclei was precipitated. The nuclear fraction was reacted with 50 µM of extraction buffer (20 mM HEPES-KOH, pH 7.9, 10% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 µM leupeptin and 1 µM aprotinin) on ice with agitation for 30 min. The supernatant obtained after centrifugation at 15,000 g for 5 min was stored in aliquots at -80°C (nuclear extract).

Electrophoretic mobility shift assay (EMSA). Nuclear extract (5 µg) was reacted for 30 min with 0.5 pmol of digoxigenin (DIG)-labeled double-stranded AP-1 oligonucleotide probe (5'-GGCTTGATGACTCAGCCGGAA-3' and 3'-GCGAAC TACTGAGTCGGCCTT-5') in a 20 µl reaction containing 20 mM HEPES-KOH, pH 7.9, 10% glycerol, 20 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 µg of BSA and 0.5 µg of poly (dI-dC) (Amersham Bioscience, Piscataway, NJ). The probes were end-labeled with DIG-11-ddUTP (Roche). Supershift assay was performed by adding 1 µg of anti-Fos Ab (sc-235; Santa-Cruz Biotechnology, Santa Cruz, CA), anti-c-Jun Ab (Ab-2: Oncogene Science, Uniondale, NY), anti-JunB Ab (sc-46; Santa Cruz), or anti-JunD Ab (sc-74; Santa Cruz). Samples were run on a 4% polyacrylamide gel using 1X TGE buffer (50 mM Tris, 380 mM grycine and 2 mM EDTA), and transferred to a positively charged nylon membrane (Roche). DIG-labeled oligonucleotides were visualized by incubating with alkaline phosphatase-labeled $F(ab)_2$ anti-DIG antibodies, followed by chemiluminescence reaction with 100 µg/ml CSPD substrates (Tropix, Inc., Bedford, MA).

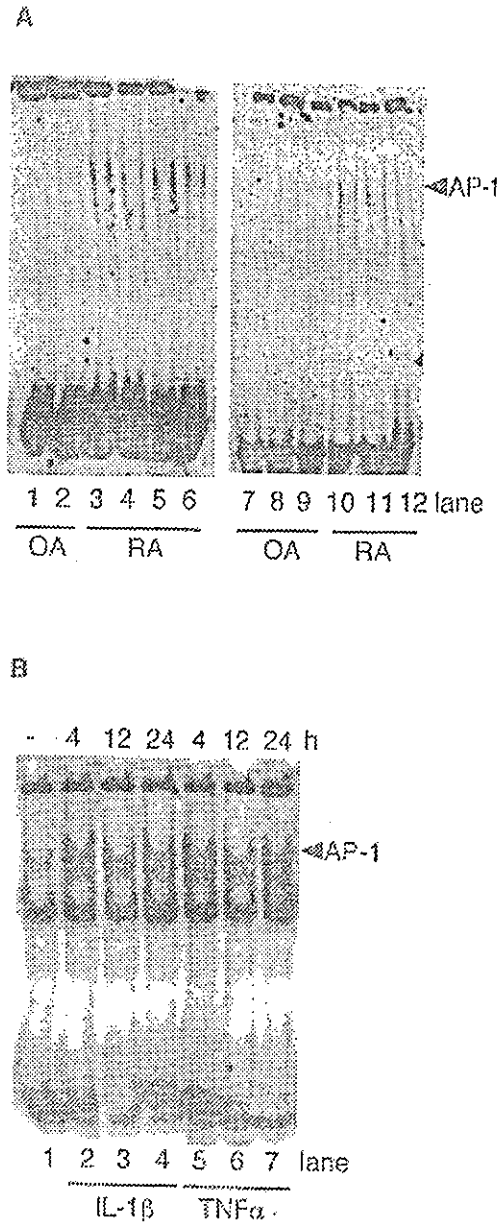

Figure 1. AP-1 binding activity in rheumatoid synovial cells. (A) EMSA for AP-1 binding comparing the nuclear extracts of synovial cells of patients with rheumatoid arthritis (RA) (n=7) and osteoarthritis (OA) (n=5). (B) EMSA for AP-1 binding under stimulation with inflammatory cytokines. Cultured rheumatoid synovial cells ($3x10^5$) stimulated with 10 ng/ml of IL-1ß (lanes 2-4) or 20 ng/ml of TNFα (lanes 5-7) for 4, 12 or 24 h were used. The data represent 4 independent experiments.

Western blotting. Cell lysates were separated using 5-15% SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA). After blocking with 5% skim milk, the membrane was incubated with rabbit IgG anti-HSP90 Ab (sc-7947; Santa Cruz), anti-Fos Ab (Santa Cruz), anti-JunD Ab (Santa Cruz) for 2 h. The membrane was then reacted with horseradish peroxidase-conjugated anti-rabbit Ig Ab (NA934; Amersham Bioscience) and bound antibodies were visualized using the ECL system (Amersham Bioscience).

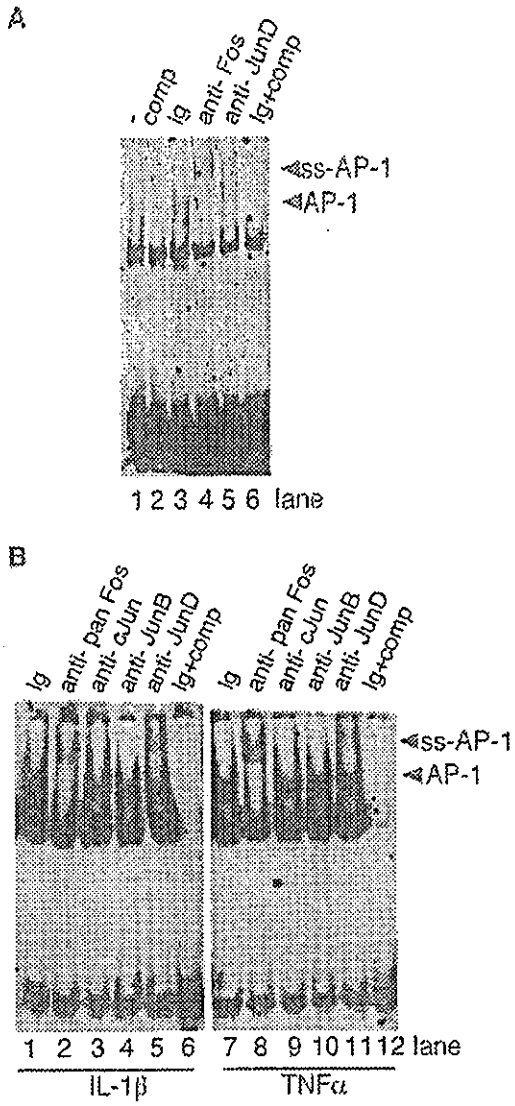

Figure 2. Supershift experiment for AP-1 in rheumatoid synovial cells. (A) Supershift experiment using specific antibodies, and competition experiment (comp) using unlabeled oligonucleotides. The bands for AP-1 and supershifted (SS) AP-1 shown with arrowheads. The data represent 5 independent experiments. (B) Supershift experiment under cytokine stimulation. The nuclear extracts obtained 6 h after stimulation with IL-1ß (lanes 1-6) or TNFα (lanes 7-12) were reacted with specific antibodies for supershift experiments. Competition assays in lanes 6 and 12. The data represent 5 independent experiments.

Results

AP-1 binding activity in RA synovial cells. The AP-1 binding activity in the nuclear extracts of rheumatoid synovial cells was compared with those of patients with OA. EMSA indicated that all 7 patients with RA showed AP-1 binding activity, whereas almost no binding was observed in patients with OA (Fig. 1A).

AP-1 binding activity is increased by inflammatory cytokines. Rheumatoid synovial cells were stimulated with inflammatory cytokines including IL-1ß and TNFα or left untreated for various time periods (4, 12, and 24 h), and the AP-1 binding activity was measured by EMSA. The AP-1 binding activity in rheumatoid synovial cells was significantly increased after stimulation with IL-1ß or TNFα (Fig.1B). The AP-1 binding peaked at 4 h and was then increased at least until 24 h after stimulation.

Supershift of Fos and JunD. Supershift experiments for AP-1 family members in the nuclear extracts of untreated or cytokine-stimulated rheumatoid synovial cells were performed using anti-Fos, anti-c-Jun, anti-JunB, and anti-JunD Abs. While only the supershift for c-Fos was observed under unstimulated conditions (Fig. 2A), supershift for both Fos and JunD proteins was observed after cytokine stimulation (Fig. 2B).

Suppression of AP-1 binding activity by specific inhibitors of HSP90. Rheumatoid synovial cells were treated either with 10 and 100 nM of geldanamycin, 1 and 10 nM of radicicol, or 10 and 100 nM of herbimycin A, and the cells were cultured with 20 ng/ml of TNFα for 6 h. The AP-1 binding activity was then compared. EMSA showed that pretreatment with as low as 10 nM of geldanamycin suppressed the increase of AP-1 binding activity in rheumatoid synovial cells (Fig. 3A, lane 3 and 4). Western blot experiments showed that the amount of cellular HSP90, Fos and JunD proteins remained unchanged irrespective of the treatments with 10 or 100 nM of geldanamycin (Fig. 4A and B, lanes 2 and 3). Further, AP-1 binding activity was also specifically inhibited by 10 nM of radicicol (Fig. 3B, lane 4) or 100 nM of herbimycin A (Fig. 3B, lane 6), while HSP90 was not decreased by the treatments (Fig. 4B).

Discussion

The results showed that AP-1 binding activity was basically higher and was further up-regulated in rheumatoid synovial cell upon stimulation with IL-1ß or TNFα, ubiquitous inflammatory cytokines elevated in sera and joint fluids of rheumatoid patients (19,20). The finding is in line with previous findings of up-regulation of c-Fos/AP-1 in arthritic disease conditions such as RA (6,21,22). As to protein composition of AP-1 dimer, we found that JunD, but not c-Jun or JunB, was specifically increased in conjunction with Fos under inflammatory cytokine stimulation, and that Fos homodimer was the major component of the AP-1 in unstimulated rheumatoid synovial cells. Previous studies have shown that c-Fos was the major component of Fos protein expressed in RA (23). Further, JunB and JunD are two major subtypes of Jun family members expressed in T cells (24,25), in which JunD is particularly expressed constantly in the cells (26). JunD is also the major component of Jun family members found in chondrocytes. JunD RNA is expressed in rheumatoid synovial cells (27). While JunD homodimer and JunB homodimer both act rather inhibitorily (28,29), Fos/JunD heterodimer has been shown to be functionally active (30,31).

We found that DNA binding activity of AP-1 was specifically inhibited by geldanamycin and other specific inhibitors of HSP90 including radicicol or herbimycin A in rheumatoid synovial cells. Further, we found that the amount of HSP90 was not decreased by the treatment with geldanamycin. Since geldanamycin specifically inhibits the function of HSP90 (14,17), it appears that functional activity, but not mere

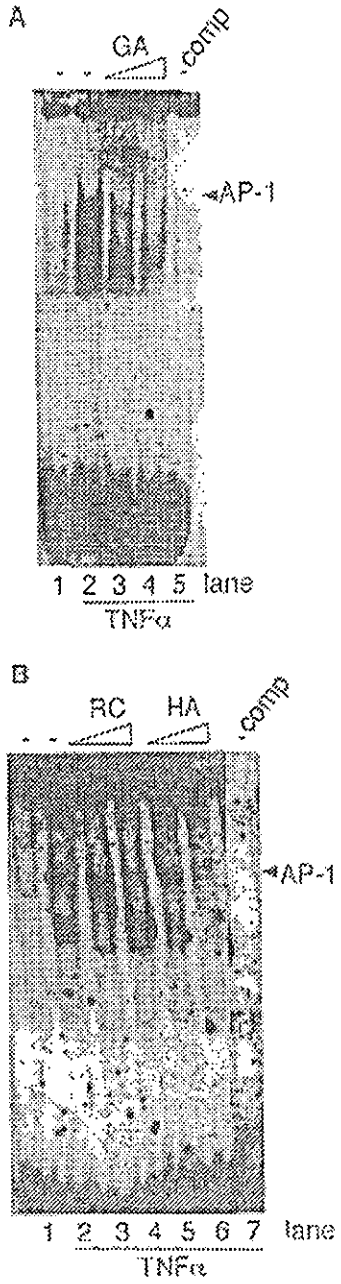

Figure 3. EMSA for AP-1 binding activity with HSP90 specific inhibitors in rheumatoid synovial cells. (A) EMSA for AP-1 binding and inhibitor geldanamycin treatment in rheumatoid synovial cells. Cultured synovial cells ($3x10^5$) were treated with 10 nM (lane 3), 100 nM (lane 4) of geldanamycin (GA), inhibitor of chaperon function of HSP90, for 12 h or left untreated (lanes 1 and 2). Cells were subsequently stimulated with 20 ng/ml of TNFα for 6 h (lanes 2-5). Competition assays done by unlabeled oligonucleotides (lane 5). The data represent 5 independent experiments. (B) EMSA for AP-1 binding and inhibitors radicicol and herbimycin A in rheumatoid synovial cells. Cultured synovial cells ($3x10^5$) were treated with 1 nM (lane 3) or 10 nM (lane 4) of radicicol (RC), 10 nM (lane 5) or 100 nM (lane 6) of herbimycin A (HA) for 12 h or left untreated. Cells were subsequently stimulated with 20 ng/ml of TNFα for 6 h (lanes 2-6). The data represent 5 independent experiments.

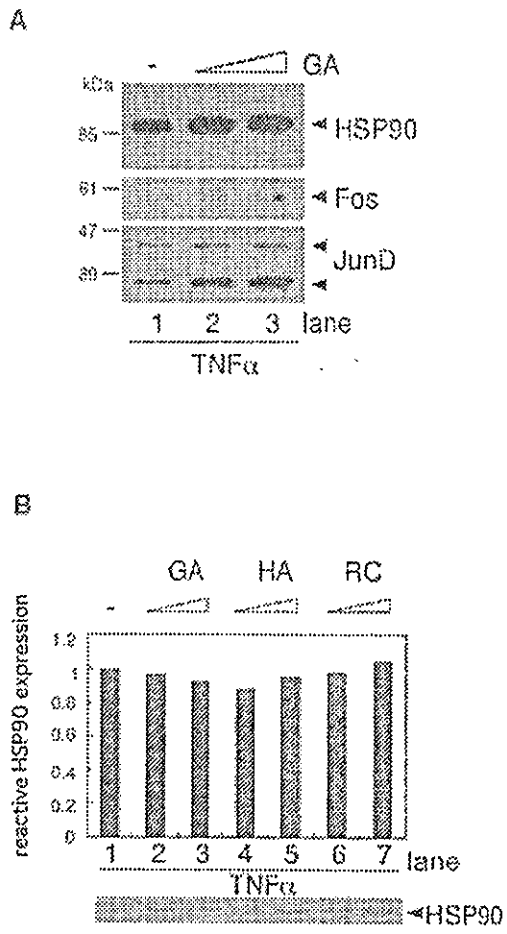

Figure 4. Western blotting for AP-1 and HSP90 with HSP90 specific inhibitors in rheumatoid synovial cells. (A) Western blotting for HSP90, Fos, and JunD proteins. Cultured RA synovial cells were treated with 10 nM (lane 2) or 100 nM (lane 3) of geldanamycin for 12 h or left untreated (lane 1). Cells were subsequently stimulated with 20 ng/ml TNFα for 6 h. Cell extracts assayed by Western blotting using anti-HSP90 Ab (upper panel), anti-Fos Ab (middle panel), or anti-JunD Ab (lower panel). The figure represents 4 independent experiments using different rheumatoid synovial cells. (B) Western blotting for HSP90 with inhibitors, geldanamycin, radicicol, and herbimycin A, in rheumatoid synovial cells. Cultured RA synovial cells were treated with 10 nM (lane 2) or 100 nM (lane 3) of geldanamycin (GA), 10 nM (lane 4) or 100 nM (lane 5) of herbimycin A (HA), or 1 nM (lane 6) or 10 nM (lane 7) of radicicol (RC) for 12 h or left untreated (lane 1). Cells were subsequently stimulated with 20 ng/ml of TNFα for 6 h. HSP90 expression was quantified by densitometry and compared (upper graph). The data represent 2 independent experiments.

presence, of HSP90 is required for AP-1 binding in rheumatoid synovial cells. Previous studies have shown that one of the major inflammatory cytokines, IL-6, up-regulates transactivation of HSP90 (32) and that IgG antibody against HSP90 is detectable in rheumatoid patients with joint erosion (33). Further, geldanamycin suppresses progression of adjuvant-induced arthritis (34). Thus, it is likely that HSP90 modulates transactivation of the genes such as IL-1ß, IL-6, TNFα and matrix metalloproteinases indirectly through AP-1 binding (35).

Previous studies have shown that HSP90 does not increase the amount of AP-1 proteins in human lung carcinoma A549 cells when stimulated with TNFα or phorbol 12-myristate 13-acetate (35). However, HSP90 up-regulated the DNA binding activity, i.e., the function of AP-1 (Fos/JunD). While JunD was composed of two isoforms, 38 and 43 kDa, as deduced from two different translational start codons contained in *JunD* gene as found in this and other studies (36), we found that the amounts of JunD and Fos was unchanged or rather slightly increased by the treatment with geldanamycin (Fig. 2B). Thus, geldanamycin appears to inhibit AP-1

binding activity by functionally inactivating HSP90. While the relationship between AP-1 protein and geldanamycin in relation to HSP90 has been pointed out in the literature (35), the evidence obtained so far did not indicate that HSP90 directly bind AP-1. Molecular mechanism of the action of HSP90 on AP-1 should be clarified in future.

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Estrogen specifically stimulates expression and production of osteoprotegerin from rheumatoid synovial fibroblasts

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Abstract. We studied the effects of estrogen on human fibroblast-like synovial cells in rheumatoid arthritis (RA-FLS) focusing on receptor activator of NF-κB ligand (RANKL) and its decoy receptor osteoprotegerin (OPG), the osteoclast formation and function regulators that have a substantial role in bone erosion of RA. Estrogen influences osteoporosis and the onset of RA clinically. The cellular responses of RA-FLS to estrogen are initiated via two high-affinity estrogen receptors (ERs). Culture of RA-FLS in the presence of 10^{-6} M 17ß-estradiol (E2) increased expression of estrogen receptor (ER)-α, but not ER-ß. OPG mRNA expression was significantly increased, whereas RANKL mRNA was unaffected. E2 treatment also significantly increased the amount of OPG released in the culture supernatant. The increase of OPG and ER-α was specifically antagonized by the pure estrogen antagonist ICI 182780. Tamoxifen, a selective ER moderator, did not increase OPG. The results indicate that estrogen stimulates secretion of OPG from RA-FLS by acting on ER-α, which likely prevents bone erosion in RA.

Introduction

Periarticular bone erosion is the beginning of devastating rheumatic joint destruction (1,2). Bone-resorbing osteoclasts formed in the synovial tissues have important roles in rheumatoid bone destruction (3-6). Molecules important for osteoclastogenesis, including receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG), are expressed in human fibroblast-like synovial cells of rheumatoid arthritis (RA-FLS) (6,7). RANKL is a membrane-bound molecule that binds to the osteoclast precursors to regulate osteoclast formation (8-10). OPG, a soluble tumor necrosis factor (TNF) receptor-like molecule termed TNF receptor superfamily 11B, is a naturally occurring RANKL inhibitor. OPG competitively binds RANKL to inhibit the action of the receptor activator of NF-κB (RANK) both *in vivo* and *in vitro*, which prevents osteoclastic bone resorption (9-11). Hence, RANKL expressed on synovial fibroblasts induces rheumatoid bone erosion by generating periarticular osteoclasts, and this process is inhibited by endogenous and exogenous OPG (6).

The effect of sex hormones cannot be dismissed in the pathogenesis of RA (12,13), because RA is predominant in premenopausal women, whereas the prevalence of RA is almost equal in older men and women (14,15). The clinical severity and symptoms of RA are under the influence of the hormonal environments, including pregnancy, delivery, and menstrual cycles (14,16,17). Among sex hormones, estrogen is essential for bone metabolism. Estrogens diffuse in and out of cells to bind nuclear estrogen receptor (ER) proteins. The effects of estrogens are mediated by two distinct nuclear receptors, ER-α and ER-ß, encoded by separate genes and located on different chromosomes (18-21). ER-α and ER-ß are expressed in RA-FLS (22), and estrogen inhibits bone resorption by stimulating OPG in osteoblasts and mouse stromal cells (23,24).

We studied the effect of 17ß-estradiol (E2) on the expression of OPG and RANKL in RA-FLS, in relation to the expression of ER-α and ER-ß.

Materials and methods

Cell preparation. Synovial tissues were obtained during joint surgery from patients with RA fulfilling the criteria of the American College of Rheumatology (25) and OA, in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. Tissues were minced and digested in a solution containing 0.2% collagenase (Sigma Chemical Co., St. Louis, MO) and 0.25% trypsin-ethylenediamine tetraacetic acid (trypsin-EDTA; Difco Laboratories, Detroit, MI) at 37°C for 2 h. Dissociated cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BR, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Biowhittaker, Walkersville, ML) and 100 U/ml penicillin-streptomycin in a tissue culture flask (Corning Inc., Corning, NY) at a cell density of 1.0×10^5 cells/cm^2. After overnight

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Key words: estrogen, estrogen receptor, osteoprotegerin, receptor activator of NF-κB ligand, rheumatoid arthritis, synovial cell