Diacerein は欧州では，以前より関節痛を軽減させる symptom modifying drug として臨床的に使用されてきたが，総計 507 名の股 OA 患者に対する 3 年間にわたる投与の結果，X 線上の関節裂隙狭小化進行を有意に抑制し得たと報告されている[18]。臨床的に使用されている薬剤のなかで，structure modifying drug としての可能性をもつ数少ない候補の一つである。なお，抗 TNF-α モノクローナル抗体を OA モデルに使用すると軟骨破壊が有意に抑制されるという報告があるが，有病率の高く生命予後の良好な OA に抗 TNF- α モノクローナル抗体などのクリティカルな合併症を有する薬剤を使用することには様々な議論がある。

3　おわりに

OA は高齢者における運動器の most common disease であるが，薬物療法に効果的なものが少ない。その進行を遅らせる真の抗 OA 薬である structure modifying drug と言えるものは厳密にはまだない。骨粗鬆症の領域ではビスフォスフォネート製剤という薬剤の開発によって，その研究が飛躍的に発展した。OA においても break through となる薬剤の開発が待たれる。

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LIGHT Induces Cell Proliferation and Inflammatory Responses of Rheumatoid Arthritis Synovial Fibroblasts via Lymphotoxin ß Receptor

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ABSTRACT. ***Objective.*** To investigate the effects of LIGHT (lymphotoxin-like, exhibits inducible expression and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes) on the proliferation and gene expression of fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA).

Methods. We measured LIGHT levels in RA synovial fluids (SF) by ELISA, and compared them with those in osteoarthritis (OA) SF. Levels of LIGHT and its receptors in RA-FLS and synovium were assessed using real-time quantitative polymerase chain reaction (PCR). RA-FLS proliferation was examined by a bromodeoxyuridine assay. Expression of intercellular adhesion molecule-1 (ICAM-1) and several chemokines, such as interleukin 8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1α (MIP-1α), was examined by real-time quantitative PCR, ELISA, and flow cytometry. The effects of LIGHT on nuclear factor-κB (NF-κB) activation were investigated using immunofluorescence and Western blotting.

Results. LIGHT was upregulated in both SF and synovium of RA patients compared with OA patients. Herpes virus entry mediator (HVEM) and lymphotoxin ß receptor (LTßR), but not LIGHT, were detected in RA-FLS. LIGHT significantly promoted RA-FLS proliferation and induced expression of MCP-1, IL-8, MIP-1α, and ICAM-1 by RA-FLS. As well, LTßR silencing RNA (siRNA), but not HVEM siRNA, inhibited these effects of LIGHT. LIGHT induced IκBa degradation and NF-κB translocation, and a NF-κB inhibitor suppressed the effects of LIGHT on RA-FLS.

Conclusion. Our findings suggest that LIGHT signaling via LTßR plays an important role in the pathogenesis of RA by affecting key processes such as the proliferation and activation of RA-FLS. Regulation of LIGHT-LTßR signaling may represent a new therapeutic target for RA treatment. (J Rheumatol First Release xxxxx)

Key Indexing Terms:
RHEUMATOID ARTHRITIS INFLAMMATION SYNOVIOCYTES

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial hyperplasia and progressive destruction of cartilage and bone. Fibroblast-like synoviocytes (FLS), an important component of the synovial lining in joints, proliferate aggressively to form a pannus causing irreversible joint damage. In RA synovial tissue, activated FLS and infiltrating macrophages and lymphocytes produce inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin 1ß (IL-1ß), and IL-6, that play important roles in the pathogenesis of RA[1,2]. These cytokines have been shown to not only directly promote FLS proliferation leading to pannus formation[3], but also to induce the expression of inflammatory cytokines, chemokines, and adhesion molecules, which further recruit inflammatory leukocytes and perpetuate inflammatory responses.

LIGHT (lymphotoxin-like, exhibits inducible expression and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes) is a recently identified type-2 transmembrane glycoprotein of the TNF ligand superfamily (TNFSF14)[4]. LIGHT is expressed on activated T lymphocytes[4,5], monocytes[6], granulocytes[6], and immature dendritic cells[7]. LIGHT signaling is transduced via 2 members of the TNFR family,

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Accepted for publication January 10, 2008.

herpes virus entry mediator (HVEM, TNFRSF14) and lymphotoxin ß receptor (LTßR, TNFRSF3). HVEM is expressed prominently on monocytes, dendritic cells, and lymphocytes[5,8-10], whereas LTßR is expressed on many cell types with the exception of lymphocytes[4,6,11]. LIGHT has been shown to regulate cell proliferation[7,12,13] and apoptosis[6,14] to induce the secretion of various cytokines, and to augment the expression of adhesion molecules[12,15-17]. Recently, Fava, *et al* reported that LTßR-Ig protein blocked the induction of experimental arthritis in mice[18]. Moreover, LIGHT induced the expression of inflammatory cytokines in macrophages from RA synovial fluid (SF)[19]. These studies suggest that LIGHT may be an important inflammatory cytokine in the development of RA. However, the effect of LIGHT on RA-FLS has not yet been analyzed.

Our aim was to clarify the role of LIGHT in the proliferation and inflammatory response of RA-FLS. We observed that the concentrations of LIGHT in both SF and synovium were higher in patients with RA than in those with osteoarthritis (OA). In addition, LIGHT signaling via LTßR, but not HVEM, enhanced RA-FLS proliferation and induced the expression of inflammatory cytokines, chemokines, and adhesion molecules in RA-FLS through a nuclear factor-κB (NF-κB)-dependent signal transduction pathway. We suggest that activation of RA-FLS by LIGHT/LTßR signaling may play an important role in the pathogenesis of RA.

MATERIALS AND METHODS

Chemicals. Recombinant human LIGHT and platelet-derived growth factor (PDGF)-AB were obtained from R&D Systems (Minneapolis, MN, USA). Monoclonal antibodies (mAb) against human actin and NF-κB p65 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and BD Biosciences (Palo Alto, CA, USA), respectively. The mAb against IκBa was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Pyrrolidine dithiocarbamate (PDTC) was purchased from Calbiochem (La Jolla, CA, USA).

Patients and tissue samples. All patients with RA fulfilled the 1987 American College of Rheumatology (formerly, the American Rheumatism Association) criteria[20] for the diagnosis of RA. Patients with RA ranged in age from 41 to 74 years (mean ± SD 66.0 ± 12.0 yrs). Patients with OA ranged in age from 39 to 90 years (mean ± SD 64.1 ± 14.7 yrs). All patients were women. Synovial tissues were obtained from 27 patients with RA and 11 patients with OA at the time of knee prosthetic replacement surgery. RA-FLS were established from the synovia of RA patients as described[21]. RA-FLS were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, penicillin, streptomycin, and L-glutamine. RA-FLS from passages 4–9 were used for each experiment. SF were obtained by arthrocentesis from 23 RA patients and 10 OA patients and, after centrifugation at 20,000 × g for 10 min, the supernatants were collected and frozen at –80°C until used. All specimens were obtained from patients who gave written informed consent, according to the protocol approved by the institutional review board of the National Hospital Organization, Sagamihara National Hospital.

LIGHT in synovial fluids. The amount of LIGHT in SF was measured using an ELISA kit (R&D Systems) according to the manufacturer's instructions. The minimum and maximum detection levels of the ELISA were 7.8 pg/ml and 2000 pg/ml, respectively.

RNA extraction, cDNA synthesis, and real-time quantitative polymerase chain reaction (PCR) analysis. Total RNA was extracted from synovium and FLS using an RNeasy Micro kit (Qiagen). cDNA was generated from RNA using Omniscript Reverse Transcriptase (Qiagen) and used as a template for real-time quantitative PCR on a LightCycler (Roche Diagnostics). PCR was performed using SYBR Premix Ex Taq (Takara). The primers used for real-time PCR were as follows: for IL-6, 5'-AAG CCA GAG CTG TGC AGA TGA GTA-3' and 5'-TGT CCT GCA GCC ACT GGT TC-3'; for IL-8, 5'-ACA CTG CGC CAA CAC AGA AAT TA-3' and 5'-TTT GCT TGA AGT TTC ACT GGC ATC-3'; for granulocyte macrophage-colony stimulating factor (GM-CSF), 5'-CAT GAT GGC CAG CCA CTA CAA-3' and 5'-ACT GGC TCC CAG CAG TCA AAG-3'; for monocyte chemoattractant protein-1 (MCP-1), 5'-GCT CAT AGC AGC CAC CTT CAT TC-3' and 5'-GGA CAC TTG CTG CTG GTG ATT C-3'; for RANTES, 5'-ACC AGT GGC AAG TGC TCC AAC-3' and 5'-CTC CCA AGC TAG GAC AAG AGC AAG-3'; for MIP-1α, 5'-TCC GTC ACC TGC TCA GAA TCA-3' and 5'-AGC ACT GGC TGC TCG TCT CA-3'; for vascular cell adhesion molecule-1 (VCAM-1), 5'-CGT GAT CCT TGG AGC CTC AAA TA-3' and 5'-GAC GGA GTC ACC AAT CTG AGC A-3'; for intercellular adhesion molecule-1 (ICAM-1), 5'-CCT GAT GGG CAG TCA ACA GCT A-3' and 5'-ACA GCT GGC TCC CGT TTC A-3'; for GAPDH, 5'-GCA CCG TCA AGG CTG AGA AC-3' and 5'-ATG GTG GTG AAG ACG CCA GT-3'; for LIGHT, 5'-TCA CGA GGT CAA CCC AGC AG-3' and 5'-CCC AGC TGC ACC TTG GAG TAG-3'; for HVEM, 5'-TTT GCT CCA CAG TTG GCC TAA TC-3' and 5'-CAA TGA CTG TGG CCT CAC CTT C-3'; and for LTßR, 5'-ATG CTG ATG CTG GCC GTT C-3' and 5'-AGG CTC CCA GCT TCC AGC TA-3'.

PCR was performed under the following conditions: initial denaturation at 95°C for 10 s, then 40 cycles of 95°C for 5 s and 60°C for 20 s. When SYBR Green dye was used to monitor PCR, melting curves were routinely recorded to verify the singularity of the PCR product. In each sample, the level of cDNA was normalized to the level of GAPDH.

Proliferation assay. RA-FLS were seeded into a 96-well plate at a density of 5.0×10^3 cells/well. After 24 h of preculture, the cells were stimulated for 48 h by the addition of LIGHT or PDGF used as a positive control. A previous report described stimulation of RA-FLS proliferation by PDGF[22]. Bromodeoxyuridine (BrdU) was added for the last 24 h of culture, then incorporation of BrdU was measured using a cell proliferation ELISA (Roche Diagnostics) according to the manufacturer's instructions.

Measurement of cytokine and chemokine levels in culture supernatants. TNF-α, IL-1ß, IL-6, IL-8, and GM-CSF levels were measured in the supernatants of RA-FLS cultures using a Human Inflammatory Five-Plex Antibody bead kit (Biosource, Camarillo, CA, USA) according to the manufacturer's instructions, on a Luminex 100 instrument (Luminex, Austin, TX, USA). The levels of MIP-1α, MIP-1ß, MCP-1, eotaxin, and RANTES in the supernatant were measured using a Human Chemokine Five-Plex Antibody bead kit (Biosource).

Transfection of RA-FLS with silencing RNA (siRNA). All siRNA were purchased from Qiagen. The sense strand sequences of the RNA duplexes were as follows: HVEM, 5'-GGC ACU GCC UCA CAG CCA AdTdT-3'; LTßR, 5'-CAU CUA CAA UGG ACC AGU AdTdT-3'; and control siRNA 5'-UUC UCC GAA CGU GUC ACG UdTdT-3'. The day before transfection, RA-FLS were seeded into 6-well culture plates at a density of 4×10^4 cells/well, or 96-well plates at 5×10^3 cells/well, in complete medium without antibiotics. The next day, siRNA (at final concentration of 50 nM) were introduced into cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions; 24 h after transfection, media were replaced with regular culture media. The cells were then cultured for 96 h before analysis of the gene-silencing effects. mRNA levels were measured by real-time quantitative PCR analysis.

Immunofluorescence assay for NF-κB localization. To examine the nuclear translocation of NF-κB, RA-FLS were seeded at a density of 5×10^3 cells/well in 8-well Lab-Tek chamber slides (Nalgen Nunc International, Naperville, IL, USA). Cells were stimulated with 10 ng/ml LIGHT for 40 min, washed with cold PBS, and then fixed in PBS with 4% paraformaldehyde for 10 min. The cells were permeabilized with PBS and 0.1% Triton-X100 for 10 min. Nonspecific binding was prevented with blocking buffer

containing 2% goat serum diluted in PBS. The cells were incubated with mouse monoclonal anti-NF-κB p65 antibody or an isotype control for 1 h at room temperature, then with AlexaFluor 488-conjugated goat anti-mouse antibody for 30 min at room temperature. Slides were coverslipped and examined using a fluorescence microscope (Olympus, Tokyo, Japan).

Western blotting analysis. For measurement of IκBa by Western blotting, RA-FLS at a density of 1.5×10^6/well were seeded into 6-well plates in culture medium for 24 h. After incubation with 10 ng/ml LIGHT for 40 min, cells were washed twice in ice-cold PBS and lysed in 100 μl of sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris HCl, pH, 10% glycerol, 2% SDS, 5% mercaptoethanol, and 0.001% bromophenol blue). Cell lysates were separated by SDS-polyacrylamide gel electrophoresis in 12% polyacrylamide gels, and transferred onto nitrocellulose membranes (Invitrogen). After blocking, membranes were incubated with either anti-ß-actin or anti-IκBa antibody, overnight at 4°C, and then with secondary antibody conjugated to horseradish peroxidase (Dako), at room temperature for 1 h. The signals were visualized using chemiluminescence reagent (ECL; Amersham Biosciences, Little Chalfont, UK).

Statistical analysis. Comparisons of = 3 populations were made using the Kruskal-Wallis test. Comparisons of 2 independent data sets were by Mann-Whitney U-test. P values less than 0.05 were considered statistically significant.

RESULTS

Increased expression of LIGHT in SF of patients with RA. To examine whether LIGHT is involved in the pathogenesis of RA, we analyzed the level of LIGHT in SF from 23 RA patients and 10 OA patients by ELISA. SF from OA patients were used as controls, because they were not available from healthy individuals. The concentration of LIGHT in SF from RA patients was significantly higher than in those from OA patients (Figure 1). The median levels of LIGHT in SF from RA and OA patients were 108.5 pg/ml and 7.8 pg/ml, respectively.

Expression of LIGHT and its receptors in RA synovial tissue and RA-FLS. Because RA patients had more LIGHT in their SF than OA patients, we investigated whether LIGHT and its membrane-bound receptors HVEM and LTßR were expressed in the RA and OA synovial tissues. Although quantitative real-time PCR analysis revealed that mRNA expression of LIGHT in synovial tissue was significantly higher in RA patients than in OA patients (Figure 2A), HVEM and LTßR levels were not different between RA and OA patients.

Further, we investigated the mRNA expression of LIGHT, HVEM, and LTßR in RA-FLS by quantitative real-time PCR. RA-FLS from all 7 patients expressed HVEM and LTßR mRNA, and the level of LTßR mRNA was significantly higher than that of HVEM mRNA, whereas no LIGHT expression was detected (Figure 2B).

Induction of RA-FLS proliferation by LIGHT. Previous studies reported that LIGHT induces cell proliferation in T lymphocytes[7,12] and vascular smooth muscle cells[13]. Since the expression of HVEM and LTßR in RA-FLS had been confirmed, we next evaluated the effect of LIGHT on the proliferation of RA-FLS using a BrdU assay. As shown in

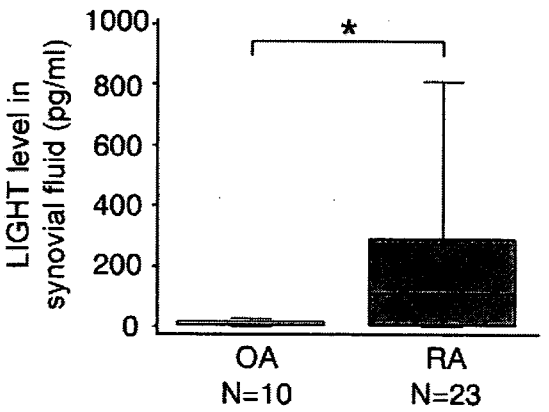

Figure 1. Levels of LIGHT in synovial fluid from RA patients and OA patients, determined by ELISA. Box plots represent 25th to 75th percentiles. Error bars represent 10th to 90th percentiles. Lines inside boxes represent the median. *p < 0.05.

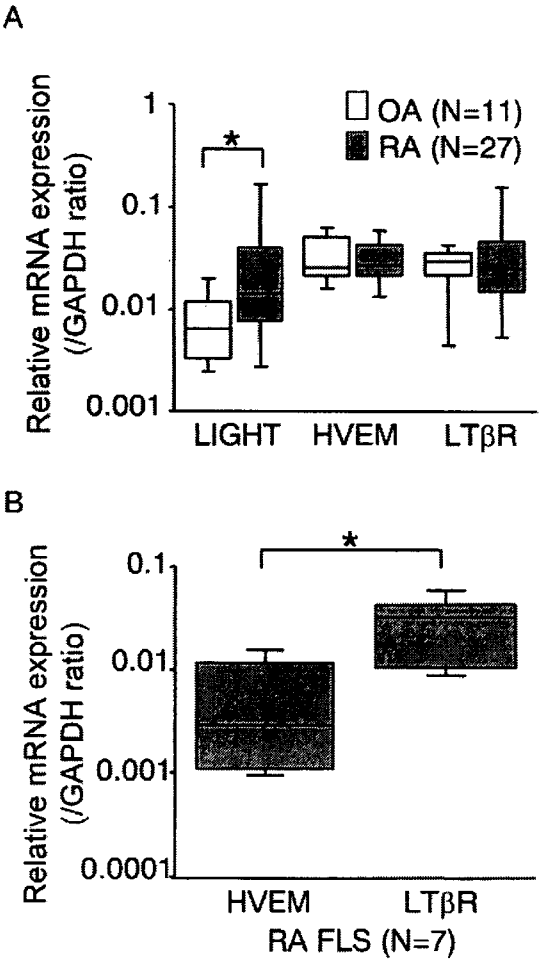

Figure 2. Expression of LIGHT, herpes virus entry mediator (HVEM), and lymphotoxin ß receptor (LTßR) mRNA in synovial tissues and fibroblast-like synoviocytes (FLS): A. In synovial tissues from RA patients and OA patients. B. In RA-FLS. Level was evaluated by real-time quantitative PCR; results are represented as relative ratios to GAPDH levels. *p < 0.05.

Figure 3A, treatment with LIGHT significantly enhanced *de novo* DNA synthesis in RA-FLS in a dose-dependent manner. LIGHT showed a stronger growth-promoting activity than PDGF, at lower concentrations.

To investigate the contributions of HVEM and LTßR to the LIGHT-induced proliferation of RA-FLS, we transfected RA-FLS with HVEM siRNA or LTßR siRNA. Quantitative real-time PCR analysis revealed that the HVEM mRNA level in HVEM siRNA-transfected RA-FLS was reduced by 75% compared with control siRNA-transfected RA-FLS (Figure 3B). Similarly, treatment of RA-FLS with LTßR siRNA led to a 75% reduction in the LTßR mRNA level compared with that in control siRNA-transfected RA-FLS (Figure 3B). Under these conditions, LIGHT-induced growth of RA-FLS was significantly decreased by LTßR siRNA, but not by HVEM siRNA, when compared with RA-FLS transfected with control siRNA (Figure 3C).

LIGHT induces expression of proinflammatory cytokines, chemokines, and adhesion molecules in FLS via LTßR. Previous studies reported that LIGHT induces secretion of various cytokines and augments the expression of adhesion molecules[13,15-17,19]. We examined the effects of LIGHT on inflammatory cytokine and chemokine production by RA-FLS. Treatment with LIGHT enhanced both mRNA and protein expression of IL-8, MCP-1, MIP-1α, and RANTES in RA-FLS, in a dose-dependent manner (Figures 4A, 4B). LIGHT induced IL-1ß, IL-6, and GM-CSF, but not TNF-α, eotaxin, or MIP-1ß (data not shown). Next, to assess whether LIGHT can induce the expression of cell-surface adhesion molecules on RA-FLS, we examined ICAM-1 and VCAM-1 expression on LIGHT-stimulated RA-FLS. LIGHT treatment significantly increased the expression of ICAM-1 mRNA in a dose-dependent manner (Figure 4C). Flow cytometry analysis revealed the augmented expression of ICAM-1 protein on the cell surface of RA-FLS stimulat-

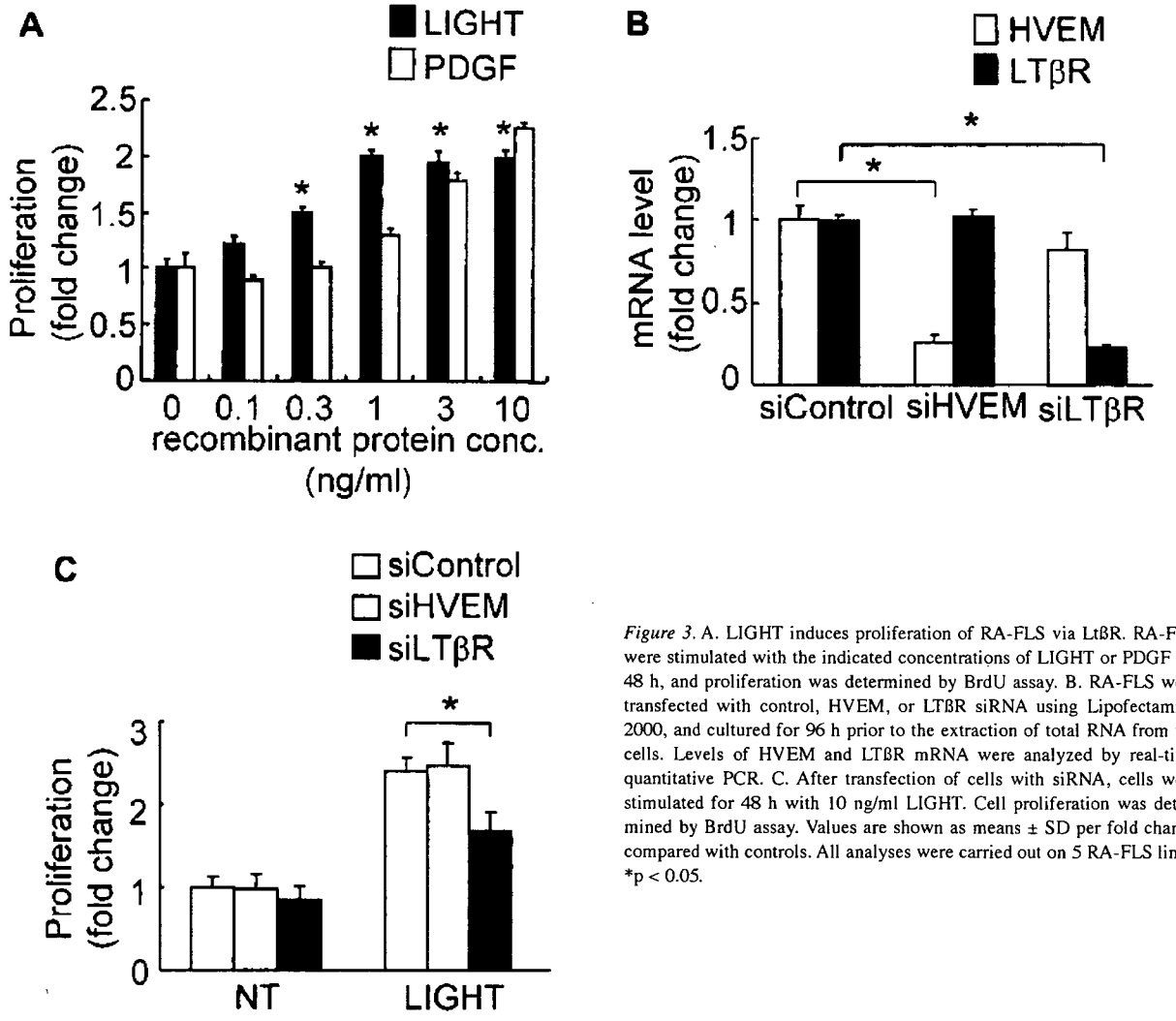

Figure 3. A. LIGHT induces proliferation of RA-FLS via LtßR. RA-FLS were stimulated with the indicated concentrations of LIGHT or PDGF for 48 h, and proliferation was determined by BrdU assay. B. RA-FLS were transfected with control, HVEM, or LTßR siRNA using Lipofectamine 2000, and cultured for 96 h prior to the extraction of total RNA from the cells. Levels of HVEM and LTßR mRNA were analyzed by real-time quantitative PCR. C. After transfection of cells with siRNA, cells were stimulated for 48 h with 10 ng/ml LIGHT. Cell proliferation was determined by BrdU assay. Values are shown as means ± SD per fold change compared with controls. All analyses were carried out on 5 RA-FLS lines. *$p < 0.05$.

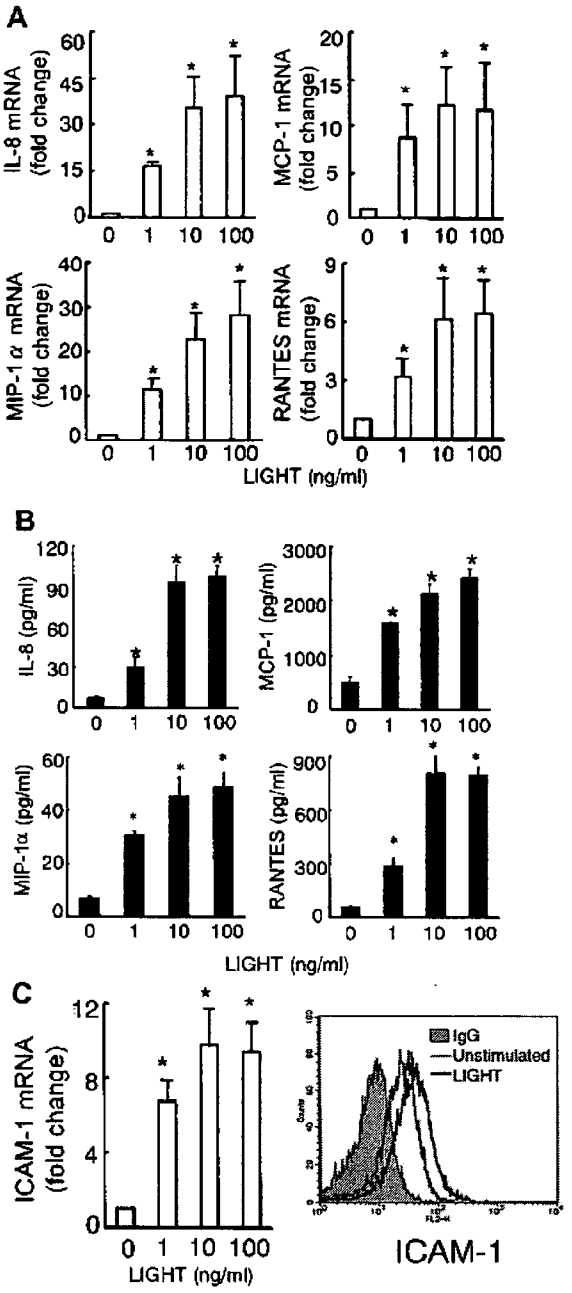

Figure 4. Upregulation of IL-8, MCP-1, MIP-1α, RANTES, and ICAM-1 expression in RA-FLS by LIGHT. A. RA-FLS were stimulated with the indicated concentrations of LIGHT for 3 h, and real-time quantitative PCR was performed to determine levels of IL-8, MCP-1, MIP-1α, and RANTES mRNA expression. Values are shown as means ± SD per fold change compared with controls. B. RA-FLS were stimulated with the indicated concentrations of LIGHT for 72 h. Concentrations of IL-8, MCP-1, MIP-1α, and RANTES in cell culture supernatants were determined by multiplex bead array assays. Values are shown as means ± SD pg/ml. C. RA-FLS were stimulated with the indicated concentrations of LIGHT for 3 h, and real-time quantitative PCR was performed to determine levels of ICAM-1 mRNA expression. ICAM-1 surface expression on RA-FLS was detected by flow cytometry after stimulation with 10 ng/ml LIGHT for 24 h. All analyses were carried out on 4 RA-FLS lines; flow cytometry profiles of one representative result are shown. *$p < 0.05$.

ed with LIGHT (Figure 4C). Similar increases in VCAM-1 mRNA and protein expression were also seen when stimulated with LIGHT (data not shown). Moreover, we investigated whether knockdown of HVEM or LTβR suppressed this series of LIGHT-induced gene expression in RA-FLS. Compared with control siRNA, LTβR siRNA, but not HVEM siRNA, significantly decreased the expression of IL-8, MCP-1, and ICAM-1 mRNA induced by LIGHT (Figure 5). Similarly, LTβR siRNA decreased the LIGHT-induced expression of IL-1ß, IL-6, GM-CSF, RANTES, and MIP-1α mRNA in FLS (data not shown).

Activation of NF-κB in RA-FLS via LTβR by LIGHT. It is known that activation of NF-κB has a key role in inflammatory disease[23]. Several studies have shown that LIGHT activates the transcription factor NF-κB in different cell types[7,9,13,24-26]. To investigate the involvement of NF-κB in LIGHT-induced gene expression, we examined the effect of the NF-κB inhibitor PDTC on the expression of IL-8, MCP-1, and ICAM-1 by real-time quantitative PCR. PDTC completely abolished the LIGHT-induced expression of IL-8, MCP-1, and ICAM-1 (Figure 6A). The LIGHT-induced expression of IL-1ß, IL-6, GM-CSF, RANTES, and MIP-1α mRNA in RA-FLS was also inhibited by PDTC treatment (data not shown). The concentration of PDTC used in these experiments had no cytotoxic effect, as demonstrated by cell viability studies using trypan blue exclusion, which showed that > 95% of cells remained viable over the entire period of the experiment (data not shown).

In the immunocytofluorescence analysis using anti-NF-κB p65 mAb, enhanced nuclear translocation of NF-κB p65 was observed in LIGHT-stimulated RA-FLS (Figure 6B). Further, Western blotting using anti-IκBa mAb showed that IκBa degradation was induced by LIGHT, and that IκBa degradation was inhibited by LTβR siRNA, but not by HVEM siRNA (Figure 6C).

DISCUSSION

We observed that LIGHT, but not HEVM or LTβR, is overexpressed in the synovial tissues of patients with RA compared with those of patients with OA. The expression of LIGHT was not detected in RA-FLS, which comprise one of the major components of the RA synovium. RA synovium is histologically characterized by prominent infiltration of macrophages and lymphocytes[27]. Although LIGHT has been supposed to be produced by activated T lymphocytes *in vitro*[4,5], a recent study reported that LIGHT was overexpressed in CD68-positive macrophages in RA synovial tissue compared with those in OA synovial tissue, and that expression levels of LIGHT were low in areas rich in lymphocytes[19]. Thus, macrophages rather than FLS and lymphocytes could be the major source of LIGHT in the RA synovium.

We further demonstrated that *in vitro*-cultured RA-FLS express HVEM and LTβR, which implies that RA-FLS are

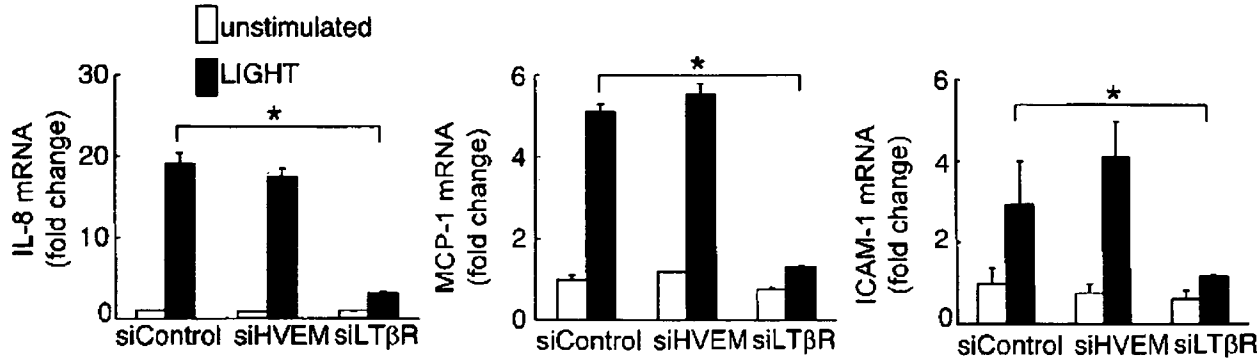

Figure 5. LIGHT-induced expression of IL-8, MCP-1, and ICAM-1 in RA-FLS via LTßR. RA-FLS were transfected with control, HVEM, or LTßR siRNA using Lipofectamine 2000. After 96 h incubation, cells were stimulated with 10 ng/ml LIGHT for an additional 3 h. Levels of IL-8, MCP-1, and ICAM-1 mRNA were analyzed by real-time quantitative PCR. Values are shown as means ± SD per fold change compared with controls. All analyses were carried out on 4 RA-FLS lines. *$p < 0.05$.

target cells of LIGHT. Indeed, we first showed that LIGHT had a stronger RA-FLS growth-promoting activity than PDGF, in lower concentrations. The proliferation of RA-FLS is one of the most critical pathological changes in RA. Thus, our findings suggest that increased expression of LIGHT might lead to the synovial hyperplasia of RA. Anticytokine therapies targeting TNF-α, IL-1ß, and IL-6 have been used to treat patients with RA, and it has been demonstrated that such treatments may suppress the accompanying bone destruction as well as the synovitis[28,29]. In addition, recent studies have indicated that LIGHT reduces Fas-mediated apoptosis in FLS[30], that LIGHT may function as a mediator of bone resorption through the induction of osteoclastogenesis[31], and that LTßR-Ig protein blocks the induction of experimental arthritis in mice[18]. Thus, a neutralizing antibody against LIGHT could be a useful tool for inhibition of synovial hyperplasia and bone destruction in RA.

The enhanced effects of LIGHT on RA-FLS proliferation were significantly inhibited by LTßR siRNA, but not by HVEM siRNA, suggesting that LTßR, rather than HVEM, is involved in the LIGHT-induced proliferation of RA-FLS. The exact mechanism by which LIGHT influences RA-FLS proliferation through LTßR is unknown. A potential mechanism underlying RA-FLS proliferation induced by LIGHT may involve cell-cycle regulators, including cyclin-dependent kinases (CDK). The mammal cell cycle is controlled by holoenzymes composed of a catalytic CDK and regulatory cyclin. The expression level of p21 was reduced in RA synovial linings and FLS compared with the level in patients with OA[32]. Overexpression of p21 or p16 by adenoviral-mediated delivery suppresses FLS growth *in vitro*[33,34]. Further, LIGHT induces cell proliferation, downregulates the CDK inhibitors p21, p27 and p53, and inversely upregulates cyclin D and Rb hyperphosphorylation in vascular smooth muscle cells[13]. Thus, it is possible that LIGHT promotes FLS proliferation by shortening the cell cycle of FLS in RA. Wang, *et al* reported that LTßR-null mice show reduced BrdU incorporation in dendritic cells[35]. This supports our claim that LTßR signaling is involved in the proliferation of RA-FLS.

We observed that LIGHT also induces the production of inflammatory cytokines and chemokines and expression of adhesion molecules on RA-FLS. Inflammatory cytokines and chemokines induce the migration of cells and release of mediators of inflammation and angiogenesis, and could be involved in the pathogenesis of RA[1,2,36]. The increased expression of ICAM-1 and VCAM-1 adhesion molecules on activated endothelial cells enhances the recruitment of monocytes, lymphocytes, and neutrophils, leading to inflammation. These findings indicate that LIGHT might play an important role in inflammation in the synovial lining layer, as well as in its hyperplasia. A recent study revealed that LIGHT upregulates the expression of ICAM-1, VCAM-1, and IL-6 in RA-FLS via NF-κB activation[30,37]. Although these reports are consistent with our present results, it has not been clear which of 2 receptors is involved in the induction of these genes in FLS. Our knockdown analysis using siRNA revealed that LIGHT induces proliferation and gene expression by signaling via LTßR, but not HVEM. Braun, *et al* have shown that LTßR is expressed on RA-FLS, and that LTa1ß2, a ligand for LTßR, induces expression of inflammatory cytokines, chemokines, and ICAM-1[38]. This supports our claim that LTßR signaling is involved in the activation of RA-FLS. The NF-κB transcription factor is certainly involved in cytokine- and chemokine-driven responses and is a point of convergence for several upstream proinflammatory pathways[23]. Indeed, NF-κB activation appears to be an important factor in RA, as the expression of NF-κB is enhanced in lining cells[39,40] and in the cartilage-pannus junction in the RA synovium[41]. In our study, treatment with PDTC blocked LIGHT-induced IL-8, MCP-1, and ICAM-1 expression, suggesting that the effects of LIGHT are mediated through NF-κB. The involvement of NF-κB in LIGHT-induced proinflammatory responses was further confirmed

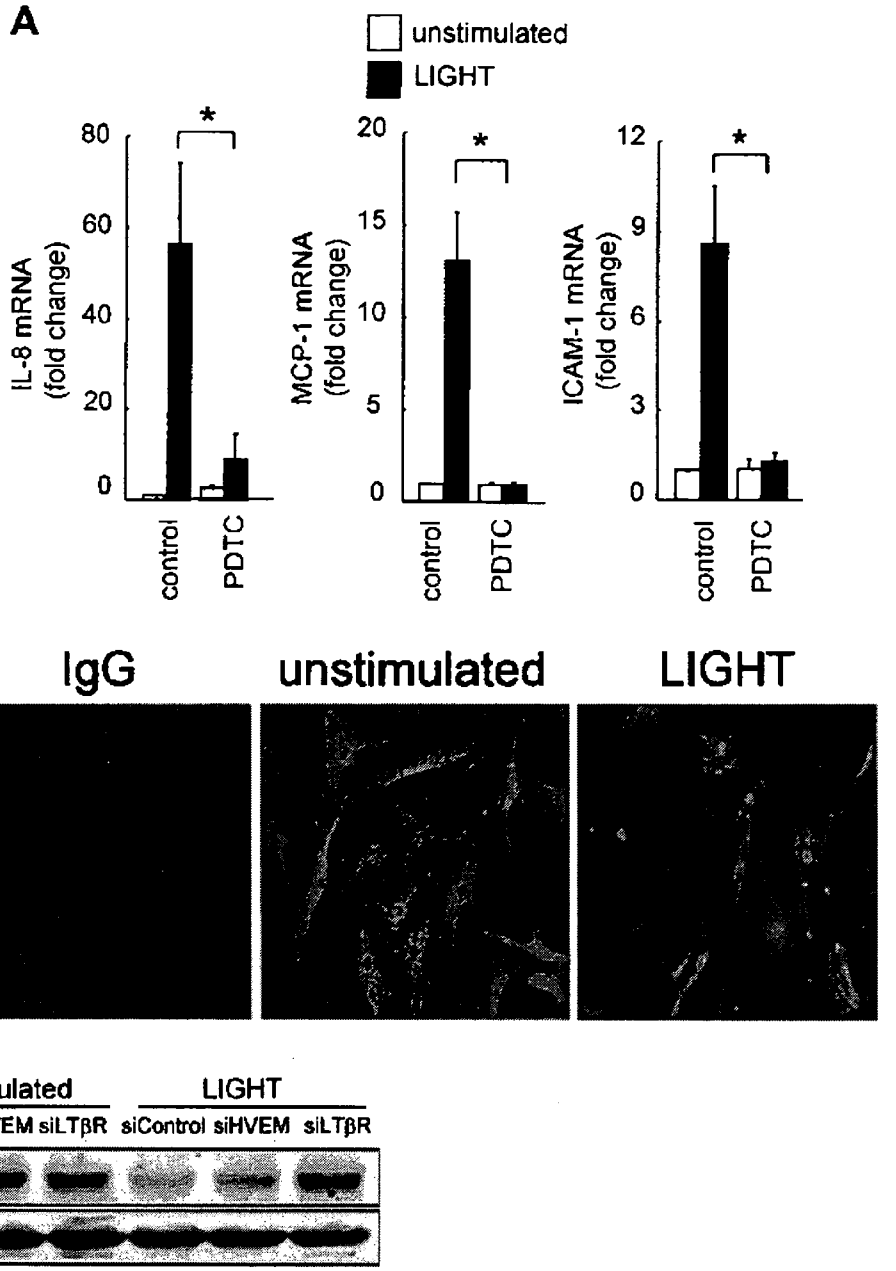

Figure 6. LIGHT-induced expression of IL-8, MCP-1, and ICAM-1 through NF-κB-mediated pathways. A. FLS were stimulated with 10 ng/ml LIGHT for 3 h with or without preincubation for 30 min with 30 μM PDTC. Levels of IL-8, MCP-1, and ICAM-1 mRNA were analyzed by real-time quantitative PCR. Values are shown as means ± SD per fold change compared with control. All analyses were carried out on 4 RA-FLS lines. *$p < 0.05$. B. Immunofluorescence staining for NF-κB p65 in RA-FLS. Control in which primary antibodies were replaced with control IgG (left panel); unstimulated RA-FLS (middle); and RA-FLS stimulated with 10 ng/ml LIGHT for 30 min (right). Results are representative of 2 experiments using 2 FLS lines. C. 96 h after siRNA transfection, cells were stimulated with 10 ng/ml LIGHT for 40 min. IκBa degradation was analyzed by immunoblotting. Results are representative of 2 experiments using 2 RA-FLS lines.

by the LIGHT-induced nuclear translocation of NF-κB p65. Moreover, LIGHT induced IκBa degradation in RA-FLS, an effect that was inhibited by LTßR siRNA, but not by HVEM siRNA. These findings are consistent with studies showing that LTßR ligation can lead to activation of NF-κB[24,42-45]. However, it is unknown why LIGHT prefers the LTßR signaling pathway in RA-FLS, even though HVEM is also expressed on these cells.

We have demonstrated that LIGHT is overexpressed in RA synovial tissues and SF. LIGHT induced increased production of inflammatory cytokines, chemokines, and adhesion molecules through NF-κB activation, as well as proliferation of RA-FLS. These findings indicate that LIGHT signaling via LTβR plays an important role in the pathogenesis of RA by affecting key processes such as the proliferation and activation of RA-FLS. Therefore, regulation of LIGHT-LTβR signaling may represent a new therapeutic target for the treatment of RA.

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Yin Yang 1 induces transcriptional activity of p73 through cooperation with E2F1

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Received 18 October 2007
Available online 1 November 2007

Abstract

The transcription factor p73 is a structural homologue of p53 and plays an important role in tumorigenesis, differentiation and development. However, the regulation of p73 pathway has not been wholly understood. Here we reported that YY1-silencing resulted in significant reductions in the activities of the p73 promoters and the endogenous p73 expression level, conversely, overexpression of YY1 could induce the activities of them. Furthermore, we showed that YY1 and E2F1 have synergistic effect on p73 promoter activity. The results of YY1-silencing and E2F1-silencing alone revealed that both factors are involved in the doxorubicin-induced activation of p73 promoter. Immunofluorescence staining and co-immunoprecipitation assays demonstrated that cooperation of YY1 and E2F1 is concomitant with physical interaction in nuclei. The results presented here suggested the cooperative transcriptional regulation of p73 by YY1 and E2F1, and might provide a new regulation mechanism by the YY1 network on tumorigenesis, differentiation and development.

Keywords: Yin Yang 1; E2F1; p73; Transcriptional activation; Synergistic effect

Yin Yang 1 (YY1) is a multifunctional transcription factor that exerts its effects on genes involved in various biological processes via its ability to initiate, activate, or repress transcription depending upon the context to which it binds, directly or indirectly via cofactor recruitments [1–3]. Today, YY1 is known to have a fundamental role in normal biological processes such as embryogenesis, differentiation, DNA replication, and cellular proliferation [3]. YY1-deficient embryos died at the time of implantation, and furthermore, its heterozygote knockout mice displayed significant growth retardation and neurological defects, indicating that YY1 plays an indispensable role in embryonic development and neuronal differentiation [4,5]. Recently, the physiologic significance of YY1 activity has also been reported to be associated with tumor biology [3,5,6]. It was known that YY1 is overexpressed in prostate cancer and sarcoma. Therefore, it is helpful to explain the cancer biological function of YY1 via studying its putative interactions with cell cycle regulators, death genes, as well as transcription factors and cofactors in the suppression or progression of various malignancies.

P73 has initially been known as a homologue of p53. They share relatively high sequence homology that includes an N-terminal transactivating (TA) domain and a central DNA binding domain. P73 has been reported to induce cell growth arrest and apoptosis in some cell lines irrespective of p53 status [7,8], and activate some p53 responsive genes, such as bax, cyclin G, IGF-BP3, and 14-3-3σ [9]. However, despite these similarities, p53 and p73 also show some fundamental differences in mechanisms and responses to DNA damage, and furthermore, in the phenotypes of their knockout mice. P73-null mice showed some defects in nervous system, which is not exhibited in p53-null mice

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doi:10.1016/j.bbrc.2007.10.145

[10,11]. On the other hand, p73-null mice do not develop spontaneous tumor formation while p53 knockout mice exhibit high susceptibility to tumorigenesis [3]. Furthermore, a very recent report showed that p73 could promote cell growth in a synergistic manner with the proto-oncogene c-Jun, and conversely, silencing p73 resulted in the reduction of growth rate and decrease of cyclin D1, indicating that p73 could act positively in tumorigenesis [12]. Therefore, p73 has a complex functions in biological processes including apoptosis, differentiation, tumorigenesis, and cell growth.

E2F1 has been identified as a regulation factor for p73 transcription. Pediconi et al. reported that responding to certain DNA damages, E2F1, but not E2F2, E2F3, or E2F4, can recruit the p73 promoter and efficiently and specifically activate its transactivation [13]. However, transcriptional regulation factors of the p73 promoter, excepting E2F1, remain unknown. Identification of other regulators that might control p73 is needed to further understand its functions and roles in tumorigenesis, differentiation and development.

Here, we identified a transcription factor, YY1, as a novel regulator of p73 transcription. Moreover, we showed that YY1 cooperates with E2F1 to induce the transcriptional activity of p73. Taken together, these findings not only shed new light on the nature of YY1's biological activities but also indicate the complex regulation mechanism of p73 in diverse biological processes.

Materials and methods

Cell cultures and chemicals. The U2OS, and HCT116 cells were obtained from the American Type Culture Collection; the SaOS2 cells were from Riken Cell Bank (Tsukuba, Japan). The U2OS and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma–Aldrich) containing 10% fetal bovine serum (FBS, Invitrogen); The HCT116 and SaOS2 cells were maintained in McCoy's 5A medium (Invitrogen) containing 10% FBS or 15% FBS, respectively.

Plasmids and constructs. The long human p73-Luciferase reporter, which contains the p73 gene fragment −4052 to +438, was generously provided by Prof. Levrero (University of Rome 'La Sapienza', Rome, Italy) [13]. The short human p73-Luciferase reporter (−857 to +71) and the human p21-Luciferase reporter were constructed by inserting each PCR products into the BglII site and HindIII site of the pGL4 basic vector (Promega).

To generate the Flag-E2F1 expression vector, pcEF9-Flag-E2F1, the coding regions of human E2F1 were amplified by PCR and inserted into the HindIII and BamHI restriction sites of pcEF9 vector [14]. For the YY1 expression vector, the coding region of human YY1 was inserted into the pcDNA3 vector (Invitrogen). For construction of the HA-YY1 vector for use in immunofluorescence staining, the YY1 coding region in pcDNA3-YY1 was excised and inserted into the BamHI and EcoRI sites of pcDNA3-HA. To construct the Flag-YY1 expression vector for use in co-immunoprecipitation experiments, the Flag-YY1 coding region fragment was generated by PCR using pcDNA3-YY1 as a template, and the PCR product was again inserted into pcDNA3 vector predigested with BamHI and EcoRI.

RNA interference. To construct siRNA expression vectors, oligonucleotides with a hairpin, overhanging sequences and terminator were synthesized, annealed and then inserted into the BspMI sites of the pcENTRhU6 vector [15]. Based on the results of applying our algorithm [16], we identified the target sequences for YY1 and E2F1 genes: siYY1-1 (GCAAGAAGAGTTACCTCAG), siYY1-2 (GGCAGAATTTGCTAG AATG), siE2F1 (GGCTGGACCTGGAAACTGA). We used a T7 siRNA expression vector, which contains a stretch of 7 thymine (Ts) terminator sequences exactly downstream of the U6 promoter, as a control.

Transient transfection and luciferase assays. The U2OS and HCT116 cells were transfected with the different p73 firefly luciferase reporters along with indicated amounts of expression vectors and *Renilla* luciferase expression vector (pRL-SV40, Promega) for transfection normalization. For knockdown experiments, HCT116 and U2OS were transfected with siRNA expression vectors, and 24 h later selection was performed by puromycin. The selected cells were transfected with p73-luciferase reporters and pRL-SV40. FuGENE6 (Roche) were used for all transfections in U2OS cells, and Lipofectamine™ 2000 (Invitrogen) for HCT116 cells. Forty-eight hours after transfection, luciferase assays were performed in triplicate using the Dual Luciferase Assay System (Promega). All relative luciferase activities were determined by calculating the ratio between firefly and *Renilla* luciferase activities, and the results were shown as means ± SDs.

Real-time RT-PCR analysis. The total RNA was isolated using TRIZOL® Reagent (Invitrogen) and treated with DNase I (Qiagen) according to the manufacturer's instructions. The total RNA (1 μg) were then reverse-transcribed using a QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was carried out using an Applied Biosystems 7500 system (Applied Biosystems) and QuantiTect® SYBR Green PCR Master Mix (Qiagen). All reactions were run in triplicate and expressional results were normalized to actin. Data were expressed as means ± SDs of triplicate wells. The primer sequences are available upon request.

Western blotting analysis. The cells were lysed in whole-cell extract buffer (50 mM Tris–HCl, pH 7.3, 10% glycerol, 250 mM sodium chloride, 2 mM EDTA, 0.1% Nonidet P-40, and 1 mM NaF) with protease inhibitors (complete cocktail; Roche), and the lysate samples proteins were electrophorated on 10% SDS–polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). After blocking, the membrane was incubated with indicated primary antibodies, and then with horseradish peroxidase (HRP)-conjugated secondary antibody IgG (Amersham Biosciences). Detections were performed with ECL Plus™ reagent (Amersham Biosciences). Antibodies used for western blotting were a rabbit polyclonal anti-E2F1 antibody (C-20, Santa Cruz), a mouse monoclonal anti-YY1 antibody (H-10, Santa Cruz), and a rabbit monoclonal anti-actin antibody (Sigma–Aldrich).

Immunofluorescence staining. The U2OS cells were seeded on cover-slips in 6-well plates and transfected with plasmids encoding HA-YY1 and E2F1. Forty-eight hours after transfection, cells were fixed for 20 min at room temperature with 10x PBS containing 4% paraformaldehyde, and permeabilized for 30 min in PBS containing 0.1% Triton X-100. After blocking, cover-slips were incubated at room temperature for 1 h in a 1:250 dilution of rat anti-HA and a 1:50 dilution of rabbit anti-E2F1 (Santa Cruz). Slides were then incubated for 1 h with a 1/1000 dilution of Alexa Fluor® 488 goat anti-rat IgG (H + L) and a 1/1000 dilution of Alexa Fluor® 568 goat anti-rabbit IgG (H + L) (Molecular Probes).

Co-immunoprecipitation. HEK293T cells were transfected with 10 μg of pcDNA3-Flag-YY1 or pcDNA3 using Lipofectamine™ 2000. Transfected cells were harvested 48 h post-transfection for Immunoprecipitation–Western blotting. Cell lysates were solved in lysis buffer (10 mM HEPES, pH 7.5, 100 mM KCl, 0.1% NP-40) with protease inhibitors on ice for 30 min and then cleared by centrifugation at 15,000 rpm. The supernatants were incubated at 4°C for 1 h with protein G-beads in the presence of 3 μg of anti-flag monoclonal antibody. Then, the immunoprecipitated proteins were subjected to western blot analysis using anti-E2F1 antibody as described above.

Results and discussion

YY1-silencing reduces the transcriptional activity of p73

Although YY1 is well known as a p53 inhibitor, recent reports have implied that YY1 has p53-independent

pathway(s) for cell cycle regulation and apoptosis [5,6]. To elucidate novel pathways of YY1, we generated siRNA vectors, siYY1-1 and siYY1-2, targeted against two different sites of YY1 for knock-down experiments. The two siRNA vectors were used to ensure elimination of off-target effect of RNAi. Western blot analysis of YY1 siRNA vectors-transfected cells revealed that both siYY1-1 and siYY1-2 could significantly suppress the endogenous level of YY1 without affecting levels of E2F1 (Fig. 1A). Furthermore, using a p21 promoter/reporter construct, which is essentially dependent on p53, we confirmed the ability of the siYY1 vectors to functionally repress endogenous YY1, which resulted in the up-regulation of p53, and subsequently, the increase of p21 promoter/reporter activities (Supplemental Fig. 1). Thus, the siRNA vectors for YY1 used here were able to knockdown and block the endogenous function of YY1 effectively and specifically.

Next, using a p73 promoter/reporter construct, we found that in contrast to the case of the p21 promoter, YY1-silencing led to a significant reduction of p73 promoter activity in both HCT116 cells and U2OS (Fig. 1B and 1C). To further confirm this finding, we performed real-time RT-PCR using total RNA extracted from YY1-silenced U2OS cells. The results showed that the reduction of p73 mRNA level was observed in the YY1-silenced U2OS cells (Fig. 1D), indicating that in contrast with p53, knockdown of YY1 was able to reduce the transcriptional activity of p73. The results implied the novel regulation of p73 by YY1.

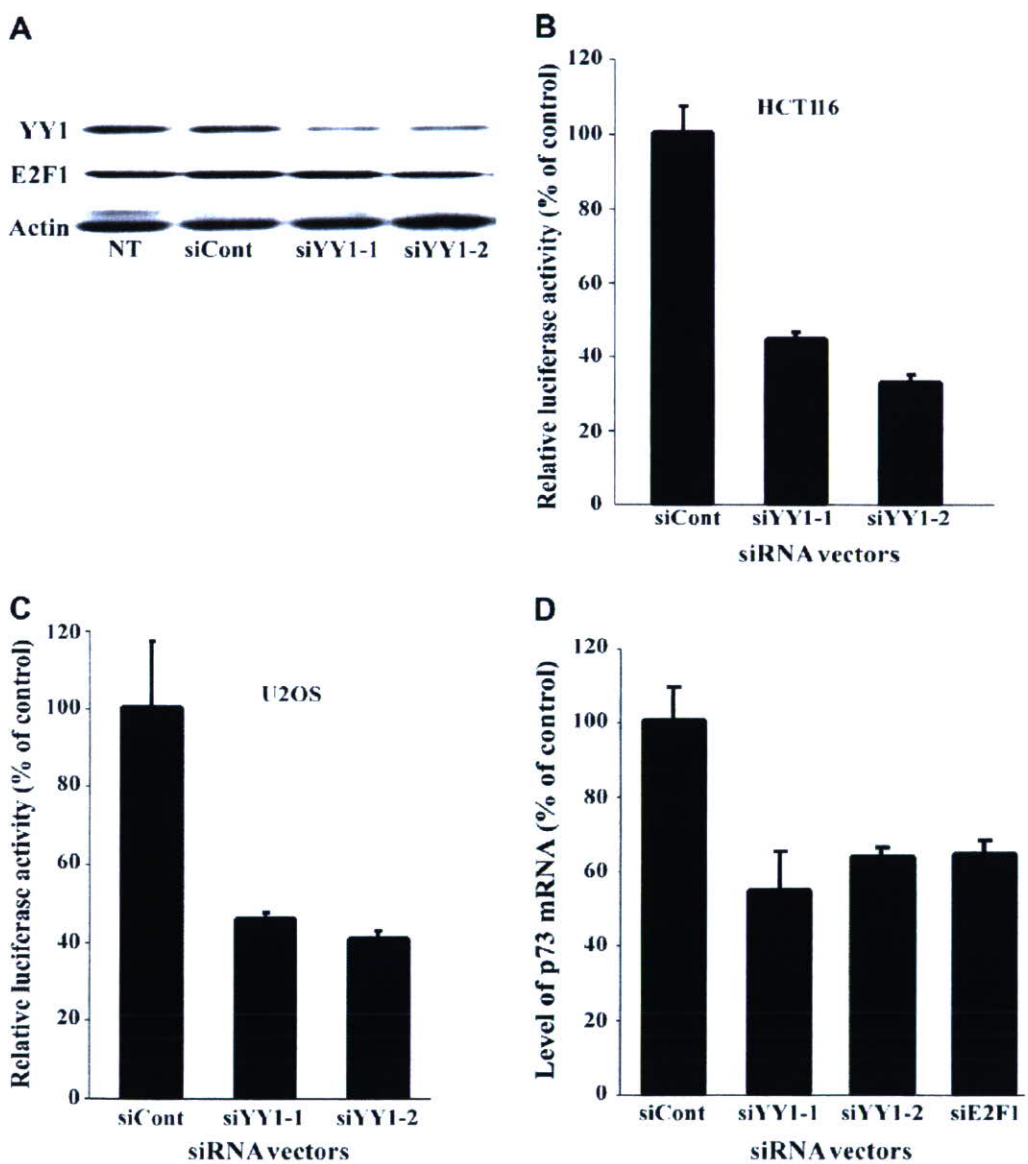

Fig. 1. The knockdown of YY1 reduces the transcriptional activity of p73. (A) HCT116 cells were transfected with siYY1-1 or siYY1-2 vectors or the siCont vector, the Western blotting was performed for detection of YY1 and E2F1. NT: non-transfected cells. (B–C) The effect of YY1-silencing on p73 promoter-driven transcription in HCT116 cells and U2OS cells. The indicated siRNA vectors-transfected HCT116 cells (B) and U2OS cells (C) were co-transfected with p73 luciferase (−4052/+438)/reporter and *Renilla* luciferase expression vector (pRL-SV40). Dual luciferase activity assay was performed 48 h after transfection. (D) Expression of p73 mRNA in YY1, E2F1-knocked down U2OS cells, determined by real-time RT-PCR analysis.

Effect of YY1 and E2F1 overexpression on the transcription activity of p73

Next, to assess the effect of YY1 overexpression and to confirm that of E2F1, a well-known p73 regulator, on p73 promoter activity, we generated a YY1-expression vector (pcDNA3-YY1) and an E2F1 expression vector (pcEF9-Flag-E2F1), and confirmed their expressions by Western blot analysis (Fig. 2A). Furthermore, the p53-inhibition activity of exogenous YY1 was also confirmed by p21 luciferase/reporter (Supplemental Fig. 2). Then, a different dose of the pcDNA3-YY1 was co-transfected into U2OS cells together with the two p73 reporters (Fig. 2B). The results were in agreement with those of knockdown experiments, as overexpression of YY1 led to the activation of the p73 promoter in a plasmid-dose dependent manner. Similar results were also obtained from SaOS2 cells, a p53 deficient human osteocarcinoma cell line (Fig. 2C), indicating that the YY1-induced p73 transcriptional activity is independent on p53 status. On the other hand, overexpressing E2F1 by co-transfecting E2F1 expression vector with the p73 promoter (−4052/+438)/reporter (Fig. 2D) or the p73 promoter (−857/+71)/reporter (Supplemental Fig. 3) resulted in the significant induction of both p73 reporters' activities, as shown previously [13]. Moreover, YY1 overexpression in U2OS cells resulted in the increase of endogenous p73 mRNA (Fig. 2E), similar with the effect of doxorubicin (Fig. 2F), which has been known to induce p73 activity via E2F1.

Collectively, the results from the knockdown and overexpression experiments for YY1 clearly identified the previously unsuspected role of YY1, i.e., the possibility that tumor activator gene YY1 up-regulates the transcriptional activity of p73. Recently, other evidences showed that spontaneous tumor formation did not develop in p73-null mice, and infrequent p73 mutations or overexpression of p73 protein was seen in a variety of tumors [17]; raising

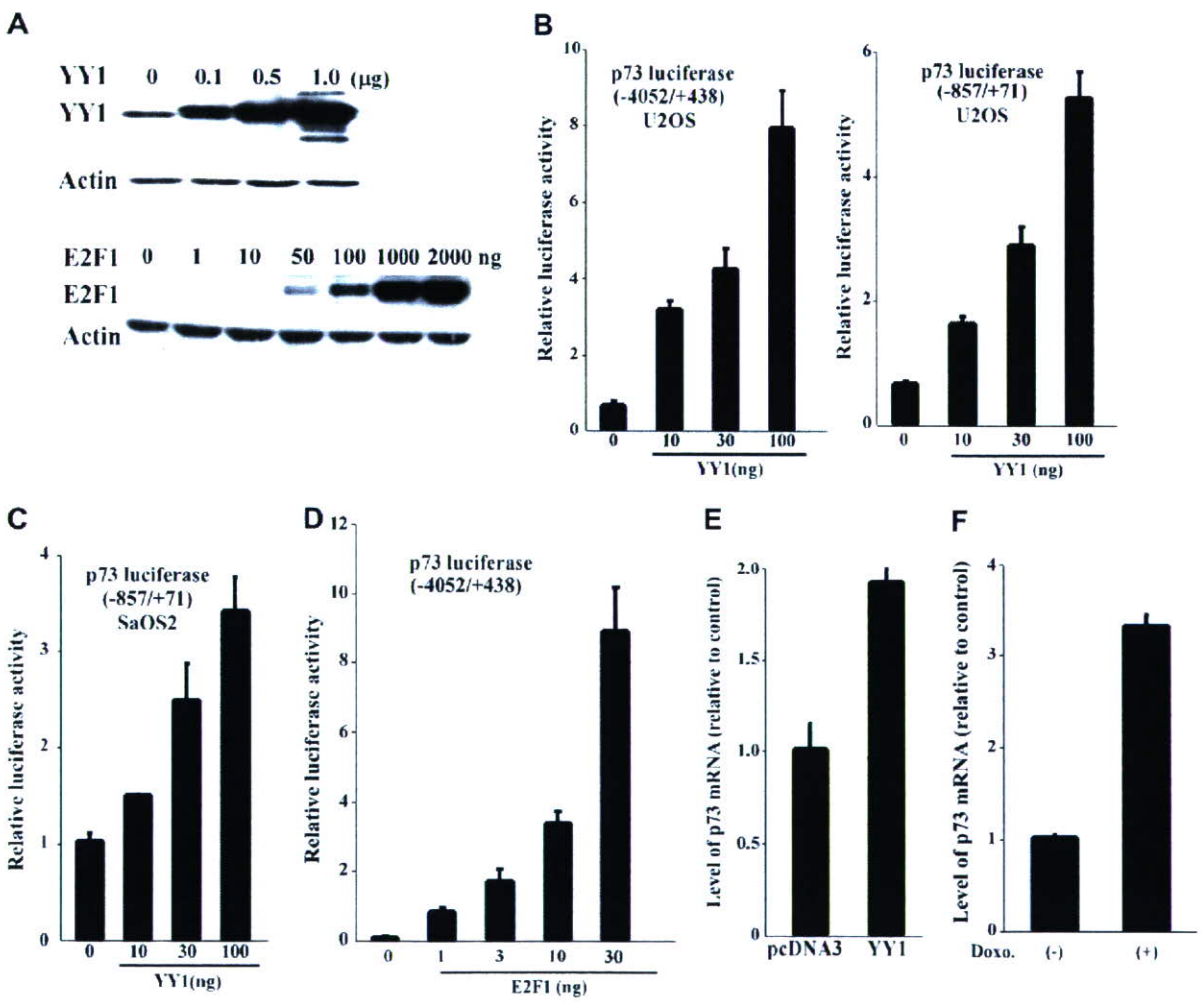

Fig. 2. The induction of p73 promoter activity by YY1 and E2F1. (A) Western blotting analysis of YY1 (upper panel) and E2F1 (lower panel) in HCT116 cells transfected with pcDNA3-YY1 or pcEF9-Flag-E2F1. (B) The effect of YY1 on the activities of p73 luciferase (−4052/+438)/reporter (left) or p73 luciferase (−857/+71)/reporter (right) in U2OS cells, determined by dual luciferase assay 48 h after transfection. (C) The effect of YY1 on the activities of p73 luciferase (−857/+71)/reporter in SaOS2 cells. (D) The effect of E2F1 on the activities of the p73 luciferase (−4052/+438)/reporter in HCT116 cells. (E) Expression of p73 mRNA in U2OS cells transfected with pcDNA3-YY1, determined by real-time RT-PCR analysis 12 h after transfection. (F) Expression of p73 mRNA in U2OS cells treated with doxorubicin (2 μM), determined by real-time RT-PCR analysis 24 h after treatment.

the question of the function of p73 as a tumor suppressor gene. A very recent work by Vikhanskaya et al. provided a potential answer to this question [12]. They found that p73 could promote cellular growth in a synergistic manner with the proto-oncogene c-Jun through AP-1 up-regulation, thus, p73 could positively act for tumorigenesis. Our results were consistent with their results. Moreover, it has also been reported that Yin Yang 1 is essential for oligodendrocyte progenitor differentiation and B-cell development [18,19]. Thus, our findings might also provide an important clue for unveiling molecular functions and mechanisms of YY1 and p73 pathway in neural differentiation and development. Therefore, we next addressed how YY1 regulates p73 gene transcription, specifically, whether there is a relationship between YY1 and E2F1 in regulating the transcriptional activity of p73.

The synergistic effect of YY1 and E2F1 on p73 transactivation

To examine whether there is cooperative regulation of p73 promoter activity by E2F1 and YY1, we performed co-transfection experiments using the p73 promoter (−4052/+438)/reporter, pcDNA3-YY1 and pEF9-E2F1 vectors. The U2OS cells co-transfected with both E2F1-expression and YY1-expression vectors showed a signification enhancement in the activity of p73 promoter compared to cells transfected with either of the vectors alone (Fig. 3A). Furthermore, in experiments with serial doses of YY1-expression vector under the constant presence of pEF9-E2F1, we found that in the presence of E2F1, the activity of p73 promoter increased in a dose-dependent manner with increasing amount of pcDNA-YY1 vector (Fig. 3B). These results demonstrated that YY1 could induce the transcriptional activity of p73 in a synergistic fashion with E2F1.

Previously, Schlisio et al. reported that E2F2 and E2F3, the members of E2F family, could interact with YY1 through mediation of the RYBP protein on the cdc6 promoter; they suggested that the interaction of E2Fs family with YY1 might determine the specificity of E2F2/E2F3 or E2F1 for different promoters [20]. In this study, we observed considerable cooperative transcriptional activation between E2F1 and YY1 on the p73 promoter. However, this functional interaction between YY1 and E2F1 might be not general, as the overexpression of YY1 did not show any significant activation of other well-defined E2F1-dependent promoters tested (DNMT1 and DHFR, data not shown), suggesting that the promoter specificity of E2F1 and E2F2/E2F3 is not simply determined by the

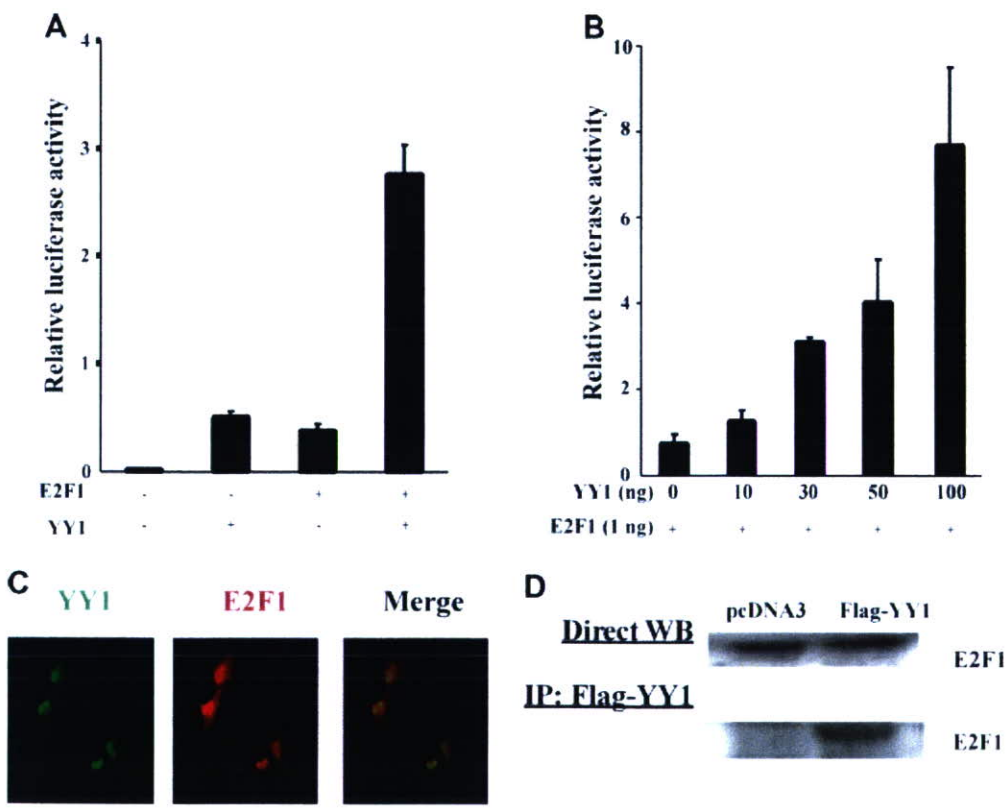

Fig. 3. The synergistic effect of YY1 and E2F1 on the transcriptional activation p73. (A) The activity of p73 luciferase (−4052/+438)/reporter in U2OS cells transfected with mock vector, pcDNA3-Flag-YY1 (50 ng), pcEF9-Flag-E2F1 (1 ng), or both pcDNA3-YY1 (50 ng) and pcEF9-Flag-E2F1 (1 ng). Dual luciferase assay was performed 48 h after transfection. (B) The activity of p73 luciferase (−4052/+438)/reporter in U2OS cells transfected with the indicated amounts of pcDNA3-YY1 and the constant amount of pcEF9-Flag-E2F1 (1 ng). (C) Immunofluorescence staining of YY1 and E2F1 in U2OS cells. (D) Co-immunoprecipitation of E2F1 and YY1 in HEK293T cells. Cells were lysed and pulled-down with anti-Flag antibody.

interaction with YY1, but might be determined by other undefined factors interacting with E2Fs and/or the epigenetic status of the promoters.

Co-localization and interaction between YY1 and E2F1

To understand the cellular and molecular mechanisms of the synergistic effects between YY1 and E2F1 on p73 promoter activation, we first examined the subcellular localization of YY1 and E2F1. As shown in Fig. 3C, YY1 was largely colocalized with E2F1 in the nucleus. Next, we examined the physical interactions between YY1 and E2F1 by performing co-immunoprecipitation experiments. The results showed that E2F1 immunoprecipitated in cells transfected with the Flag-YY1-expression vector, whereas no band was detected in cells transfected with the control vector (Fig. 3D). These observations demonstrated the direct physical association between YY1 and E2F1.

Role of YY1 in DNA damage-induced transcriptional activity of p73 promoter

As reported previously, the p73 promoter was activated E2F1-dependently by doxorubicin, a DNA damaging agent [13]. Therefore, we examined whether YY1 contributed to doxorubicin-induced p73 transcriptional activation. YY1-silenced cells showed significant reduction in the activation of the p73 promoter induced by doxorubicin (Fig. 4A, B), which was similar to that of E2F1-silencing cells.

Furthermore, p73 promoter activation by doxorubicin treatment has been reported to be critically dependent on

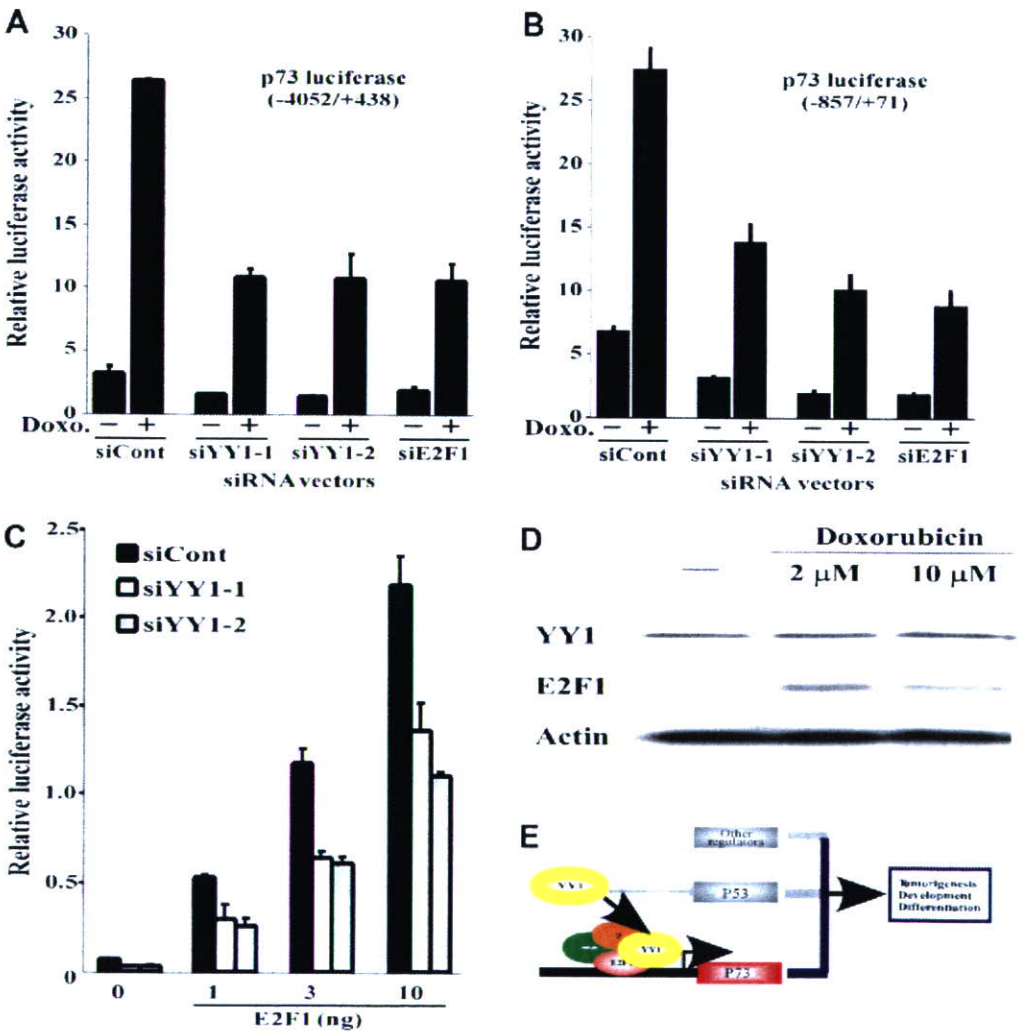

Fig. 4. Involvement of YY1 in transcriptional activation of p73 induced by doxorubicin. (A, B) The activity of p73 promoter in the YY1-silenced U2OS cells under doxorubicin treatment. siYY1-1, siYY1-2 or siE2F1 vectors-transfected U2OS cells were co-transfected with p73 luciferase (−4052/+438)/reporter (A) or (−857/+71)/reporter (B) and pRL-SV40. Twenty-four hours after transfection, U2OS cells were untreated or treated with doxorubicin (2 μM), and 24 h later, dual luciferase assay was performed. (C) The effect of YY1-silencing on p73 promoter activity induced by E2F1 in U2OS cells. The indicated siRNA vectors-transfected U2OS cells were co-transfected with p73 luciferase (−4052/+438)/reporter and increasing amount of pcEF9-Flag-E2F1. (D) Western blot analysis of E2F1 and YY1 in HCT116 cells treated by doxorubicin. (E) A model of the co-regulation of YY1 and E2F1 on p73.

E2F1 [13]. Based on the interaction of E2F1 and YY1, we next tested whether p73 promoter activation by E2F1 overexpression also showed the YY1 dependency. The YY1-knocked down cells were co-transfected with serial doses of E2F1-expression vectors and the p73 reporter vector. As shown in Fig. 4C, in YY1-silenced cells, the p73 promoter activation induced by E2F1 was significantly reduced, which was a comparable suppressive effect as that of doxorubicin-treated cells, suggesting that the YY1 signal might be constitutive during doxorubicin treatment. Together with the results of Western blot analysis (Fig. 4D), which revealed that only endogenous E2F1, not YY1 expression was induced by doxorubicin treatment, these data indicated that cooperative action between the constitutive YY1 and the inducible E2F1 contributes to the activation of the p73 promoter under the treatment with doxorubicin. Furthermore, YY1-silencing did not affect the expression of endogenous E2F1 (Fig. 1A), which also strongly suggested that YY1 induces the transcriptional activity of p73 by cooperation with E2F1, not via increasing E2F1.

Taken together, our results uncovered a novel function of YY1 on E2F1-mediated p73 regulation. We summarized the model in Fig. 4E. The fact that YY1 affects p53 family members opened up an attractive possibility that needs further investigation, that is, YY1 might function as a key integrator or modulator of various pathways in the network that includes p53 family members and regulators concerned with cancer progression, development and differentiation.

Acknowledgments

We thank Prof. Massimo Levrero at the University of Rome 'La Sapienza' for kindly providing the p73 reporter vector. This work was partially supported by a grant from the 21st Century COE Program, School of Medicine, The University of Tokyo, and a grant for Research on Psychiatric and Neurological Diseases and Mental Health, the Ministry of Health, Labour and Welfare of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.10.145.

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Photodynamic Therapy for Corneal Neovascularization Using Polymeric Micelles Encapsulating Dendrimer Porphyrins

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PURPOSE. To investigate the accumulation of new photosensitizers (PSs), dendrimer porphyrin (DP, free DP), and DP encapsulation into polymeric micelles (DP-micelle) and the efficacy of photodynamic therapy (PDT) in an experimental corneal neovascularization model in mice.

METHODS. Corneal neovascularization was induced by suturing 10-0 nylon 1 mm away from the limbal vessel in C57BL6/J mice. To determine the accumulation of free DP and DP-micelle, 10 mg/kg free DP or DP-micelle was administered by intravenous injection 4 days after suture placement. Mice were killed 1, 4, 24, and 168 hours after the injection of PS. Twenty-four hours after the administration of free DP or DP-micelle, mice were treated with a diode laser of 438-nm wavelength at 10 or 50 J/cm^2. Fluorescein angiography was performed before and 7 days after irradiation, and the area of corneal neovascularization was quantified.

RESULTS. Free DP and DP-micelle accumulated in the neovascularized area 1 hour to 24 hours after administration. Fluorescence of DP was weaker than that of DP-micelle. Neither DP-micelle nor DP could be detected in normal limbal vasculature. In the PDT experiments using PS, mean residual rates of corneal neovascularization were 10.1% in the mice treated with DP-micelle and 21.6% in the mice treated with free DP at 10 J/cm^2 ($P < 0.01$). At 50 J/cm^2, mean residual rates of corneal neovascularization were 10.6% in the mice treated with DP-micelle and 13.7% in the mice treated with free DP ($P > 0.05$). Although corneal neovascularization in PDT-treated mice exhibited significant regression compared with the control group, significant energy-related vessel regression with increasing laser energy could not be observed.

CONCLUSIONS. PDT with DP-micelle and free DP can provide efficacious treatment of corneal neovascularization. (*Invest Ophthalmol Vis Sci.* 2008;49:894-899) DOI:10.1167/iovs.07-0389

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Supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Submitted for publication April 2, 2007; revised June 27, 2007; accepted January 25, 2008.

Disclosure: **K. Sugisaki**, None; **T. Usui**, None; **N. Nishiyama**, None; **W.-D. Jang**, None; **Y. Yanagi**, None; **S. Yamagami**, None; **S. Amano**, None; **K. Kataoka**, None

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Corneal neovascularization is a major sight-threatening condition and is caused by infections, inflammation, degenerative disorders, and long-time contact lens wear.[1] This major ocular complication can lead to corneal scarring, edema, lipidic deposition, and inflammation that may not only compromise visual acuity but also decrease the success rate of subsequent penetrating keratoplasty.[2] In the clinical setting, topical corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs) remain the principal primary treatment for suppressing proliferating corneal vessels.[3] However, in corneas in which vessels have been established for extended periods, corticosteroid and NSAID treatment are ineffective. Although laser photocoagulation for corneal neovascularization has been reported,[4-7] this method achieves an inadequate effect because of the high incidence of recanalization and thermal damage to adjacent tissue.[4] The discovery of the many factors involved in corneal neovascularization and their mechanisms of action has been followed by efforts to develop new drugs specifically targeting these molecules. For example, therapy targeting vascular endothelial growth factor (VEGF) looks promising for the treatment of corneal neovascularization.[8] However, the agents tested thus far have yet to become available clinically.

Photodynamic therapy (PDT) has been introduced recently as a novel treatment for corneal neovascularization.[9] In this therapy, a photosensitizer (PS) is injected systemically and accumulates in newly formed vessels; it is then activated by mild laser excitement to liberate cytotoxic reactive oxygen species (ROS) that selectively occlude the target vessels. Although benzoporphyrin (verteporfin), a PS, is used for choroidal and corneal neovascularization clinically,[9] nonspecific binding activities of verteporfin induce skin phototoxicity in bright conditions, and patients must remain in the dark for 48 hours after injection of this drug. Hence, innovative PS should be developed for realizing safe and effective PDT.

Specific delivery of a PS to the neovasculature site might be a promising way to achieve safe and effective PDT for corneal neovascularization. Drug vehicles such as liposomes can be used for this purpose; however, the self-quenching effect of PS caused by aggregate formation could decrease the efficiency of ROS production. To solve this problem, we have recently developed dendrimer porphyrin (DP) as a novel PS for drug delivery (Fig. 1A).[10] It is assumed that the dendritic framework of DP might prevent the interactions of the center dye molecules, thereby achieving efficient ROS production even at extremely high concentrations. Indeed, encapsulation of DP into polymeric micelles (DP-micelle), which are characterized by the polyion complex core surrounded by poly(ethylene glycol) (PEG) palisades (Fig. 1C), resulted in remarkably increased photocytotoxicity.[11,12] We previously reported the review and general introduction of drug delivery of these PSs in corneal neovascularization.[13] In this study, to demonstrate the potential usefulness of DP and DP-micelle for PDT of corneal neovascularization, we investigated the accumulation of those PS

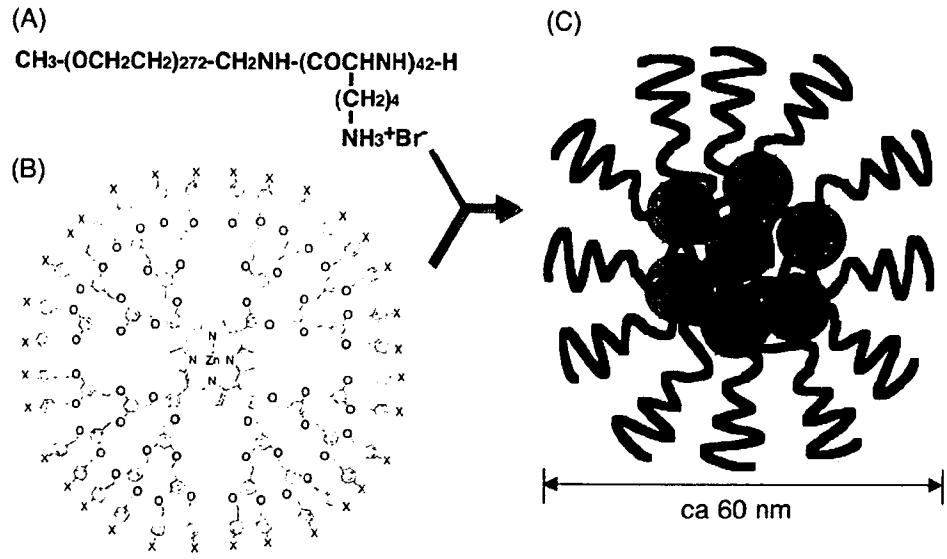

FIGURE 1. Schematic structure of polymeric micelle encapsulating DP. Chemical structures of poly(ethylene glycol) (PEG)-*b*-poly(L-lysine) (A) and ionic dendrimer porphyrin (X = COO^-) (DP) (B). DP-micelle is spontaneously formed through the electrostatic interaction between PEG-*b*-poly(L-lysine) and DP (C).

formulations and their efficacy of PDT in an experimental corneal neovascularization model in mice.

MATERIALS AND METHODS

Animals and Experimental Corneal Neovascularization

Eight-week-old male C57 BJ/6 mice were maintained with free access to food and water. All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were placed under general anesthesia by the administration (1.5 mL/kg) of a mixture of ketamine hydrochloride (Ketalar; Sankyo, Tokyo, Japan) and xylazine hydrochloride (Celactal; Bayer, Tokyo, Japan). Corneal neovascularization was induced by suturing 10-0 nylon 1 mm away from limbal vessel under microscopy. Erythromycin ophthalmic ointment was instilled immediately after the procedure.

Photosensitizers

In this study, a third-generation aryl ether dendrimer zinc porphyrin with 32 carboxyl groups on the periphery (DP) and polymeric micelles composed of the DP and PEG-*b*-poly(L-lysine) (DP-micelle) were used for PDT as a PS formulation (Fig. 1). The DP-micelle was prepared according to a previous report.[11] Both DP and DP-micelle have a maximum excitation wavelength at 433 nm. DP-micelle showed 130- to 280-fold higher photocytotoxicity against murine Lewis lung carcinoma cells compared with free DP.[11]

Accumulation of DP and DP-Micelle in Corneal Neovascularization Lesions

Four days after suture placement, DP or DP-micelle was administered by intravenous injection at the dose of 10 mg/kg, again under general anesthesia. Mice were killed 1, 4, 24, and 168 hours after the injection of PS. Before the kill, mice received intravenous BS-1 lectin conjugated with FITC (500 μg/g; Vector Laboratories, Burlingame, CA) to trace the corneal neovascularization area. Corneas were excised and flat-mounted on glass slides. Accumulations of DP or DP-micelle in vascularized areas were observed by fluorescence microscopy (Leica, Deerfield, IL) using 436-nm excitation wavelength. Fluorescence intensities were calculated using NIH Image software and were normalized by traced vascularized areas.

Photodynamic Therapy

Twenty-four hours after DP-micelle or free DP, 38 mice were treated with a diode laser (in-house built laser equipment; Topcon, Tokyo, Japan) of 438-nm wavelength at 500 mW/cm^2 for 20 or 100 seconds, for a total dose of 10 or 50 J/cm^2. The spot size was 1 mm in diameter. As controls, six mice with corneal neovascularization were irradiated without administration of photosensitizers (total dose, 50 J/cm^2). Fluorescein angiography was performed before and 7 days after treatment, and the area of corneal neovascularization was quantified using NIH Image software before and 7 days after irradiation. We defined residual ratio as follows: Residual ratio = (neovascularization area 7 days after irradiation/neovascularization area before irradiation) × 100%.

RESULTS

Accumulation of DP and DP-Micelle in Corneal Neovascularization Lesions

One hour after the administration of DP-micelle, fluorescence started to accumulate in the neovascularized area and increased until 24 hours after administration (Figs. 2A–C). DP-micelle accumulation decreased but continued after 168 hours (data not shown). After DP administration, fluorescence of DP was observed in the corneal neovascularization area, but it was weaker than that of DP-micelle group (Figs. 2D-F) and disappeared by 168 hours (data not shown). Figure 3 shows the time course of normalized fluorescence intensities of DP in the neovascularized area. DP-micelle intensities were significantly higher than those of free DP 1 hour (n = 7/each condition), 4 hours (n = 6/each condition), and 24 hours (n = 6/each condition) after administration (P = 0.032, 0.047, 0.0066; Mann-Whitney U test). Neither DP-micelle nor free DP could be detected microscopically in normal limbal vasculature or other ocular tissue such as iris, retina, and conjunctiva (data not shown).

Photodynamic Therapy

Figure 4 is a series of fluorescence angiographic images of corneal neovascularization before and 7 days after PDT in the control, DP-micelle, and DP groups. As shown in Figure 4A, there was no effect of irradiation in the control (n = 6). Seven days after irradiation at 10 J/cm^2, the mean residual ratio of