Considering these results, there is a possibility that HDAC8 expression levels were decreased more in prostate cancer than in other cancers, but the reason for this remains unknown.

In our study, 5-40% of cancer tissues overexpressed class I HDACs, compared with corresponding normal epithelium. These data show lower expression of class I HDACs, in comparison with those of previous investigations. Toh *et al* have demonstrated that 26.7-40.0% of advanced esophageal cancer tissues express HDAC1 more than does normal mucosa (15), and our data for advanced esophageal cancer tissues showed a similar result. We consider that there were a lower number of cancer tissues in which class I HDACs were overexpressed more than in normal epithelium, because our immunohistochemical study showed that the expression of class I HDACs was equal in many cancer tissues and their corresponding normal epithelia. However, our data also revealed that there were some cases of class I HDAC overexpression in cancer tissues, and HDAC inhibitors may be effective in these class I-overexpressing cancers.

HDACs are thought to play a key role in carcinogenesis. In many studies, overexpression of class I HDACs has been found in cell lines and human cancer tissues; moreover, there have been also some reports that HDAC mRNA expression is a possible prognostic factor (22,29). Therefore, HDAC inhibitors are emerging as a new class of targeted cancer therapeutics. In the current study, class I HDACs were highly expressed in many cancer tissues, but we consider that there are not so many cancer tissues in which class I HDACs are more expressed than the normal epithelium. The activity of class I HDACs is due to histone acetylation status, and HDAC complexes are related to this status (30), therefore, more detailed study, including the expression status of the HDAC complex, is required to predict the effectiveness of HDAC inhibitors. When HDAC inhibitors are used as anti-cancer agents, adjuvant administration should be conbined to avoid side effects, because in many cancers, class I HDACs are overexpressed in cancer and non-cancerous tissues.

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Inflammatory stimuli from macrophages and cancer cells synergistically promote tumor growth and angiogenesis

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The focus of the present study was whether and how infiltrating macrophages play a role in angiogenesis and the growth of cancer cells in response to the inflammatory cytokine interleukin (IL)-1β. Lewis lung carcinoma cells overexpressing IL-1β grew faster and induced greater neovascularization than a low IL-1β-expressing counterpart *in vivo*. When macrophages were depleted using clodronate liposomes, both neovascularization and tumor growth were reduced in the IL-1β-expressing tumors. Co-cultivation of IL-1β-expressing cancer cells with macrophages synergistically augmented neovascularization and the migration of vascular endothelial cells. In these co-cultures, production of the angiogenic factors vascular endothelial growth factor-A and IL-8, monocyte chemoattractant protein-1, and matrix metalloproteinase-9 were increased markedly. The production of these factors, induced by IL-1β-stimulated lung cancer cells, was blocked by a nuclear factor (NF)-κB inhibitor, and also by the knockdown of p65 (NF-κB) and c-Jun using small interference RNA, suggesting involvement of the transcription factors NF-κB and AP-1. These results demonstrated that macrophages recruited into tumors by monocyte chemoattractant protein-1 and other chemokines could play a critical role in promoting tumor growth and angiogenesis, through interactions with cancer cells mediated by inflammatory stimuli. (*Cancer Sci* 2007; 98: 2009–2018)

The common features shared by cancer and inflammation, such as macrophage infiltration, have been highlighted recently by attempts to treat cancer by targeting the inflammatory responses that are also associated with malignant tumors.[1–3] Inflammatory cells, cytokines, and chemokines in malignant tumors affect the stromal microenvironment, suggesting that inflammation and cancer may be interrelated through the angiogenic process.[4,5] During inflammation, angiogenesis often coincides with the infiltration of inflammatory cells such as neutrophils, monocytes, and macrophages, which secrete key cytokines and growth factors.[3,5]

Tumor-associated macrophages produce various pro-angiogenic cytokines and matrix-degrading proteinases, providing cancer cells and vascular endothelial cells with favorable conditions for proliferation, migration, angiogenesis, and tissue remodeling.[5–10] However, it remains unclear how TAM play this pivotal role in both tumor angiogenesis and the inflammatory response in cancer. Inflammatory cytokines such as tumor necrosis factor-α, IL-1α and IL-1β upregulate various angiogenic factors, such as VEGF, FGF-2, IL-8, MMP, PA, integrins and soluble VCAM-1 in vascular endothelial cells, cancer cells, and inflammatory cells.[11–14] We have previously reported that Sp1 and AP-1 motifs and NF-κB are prerequisites for the transcription of VEGF-A in response to inflammatory cytokines in cancer cells and vascular endothelial cells.[11,12] Expression of IL-8 is also highly inducible by inflammatory cytokines and oxygen stress, and NF-κB, AP-1, and NF-IL-6 have been implicated in this.[12,15–17]

We previously demonstrated that IL-1α and IL-1β enhance the production of VEGF and IL-8 in monocytes and macrophages, which results in the promotion of vascular endothelial cell migration.[15] Moreover, IL-1β can induce angiogenesis *in vitro* and *in vivo* by activating the COX2–prostanoid pathway following the expression of prostaglandin E2 and thromboxane A2, and this angiogenesis is blocked specifically by COX2-selective inhibitors.[18] We further demonstrated that the infiltration of macrophages is a prerequisite for IL-1β-induced angiogenesis.[18] Although this evidence has established a role for the inflammatory responses induced by TAM in the acquisition of malignant characteristics during cancer progression, exactly how TAM affect tumor angiogenesis and growth by interacting with cancer cells is still unclear. In the present study, we further investigated the mechanisms by which TAM affect tumor growth and angiogenesis.

Materials and Methods

Cell culture and reagents. The macrophage cell line U937 and human lung cancer cell line A549 were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI supplemented with 10% FBS. Another human lung cancer cell line, B203L, was established previously.[19] The LLC/IL-1β cell line and the control LLC/neo cell line were donated by Y. Saijo and T. Nukiwa (Tohoku University) and cultured in Dulbeccos's modified Eagles medium with 10% FBS.[13] Recombinant murine IL-1β was purchased from R & D Systems (Minneapolis, MN, USA). Phosphatidylcholine, cholesterol, and clodronate were purchased from Sigma-Aldrich (St Louis, MO, USA). The NF-κB inhibitor DHMEQ was used in this study.[20,21]

Effect of clodronate liposomes in the LLC/IL-1β xenograft model. Male C57BL/6 mice (Kyudo, Fukuoka, Japan) were inoculated

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Abbreviations: COX2, cyclooxygenase-2; DHMEQ, dehydroxymethylepoxiquinomicin; ELISA, enzyme-linked immunosorbent assay; EMSA, enzyme mobility shift assay; FBS, fetal bovine serum; FGF, fibroblast growth factor; HUVEC, human umbilical vein endothelial cells; IL, interleukin; LLC, Lewis lung carcinoma; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; MVD, microvascular density; NF, nuclear factor; OCT, optimal cutting temperature; PA, plasminogen activator; PDGF-BB, platelet-derived growth factor BB; PlGF, placental growth factor; RANTES, regulated upon activation, normal T expressed and secreted; siRNA, small interference RNA; TAM, tumor-associated macrophage; VEGF, vascular endothelial growth factor.

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subcutaneously with 5×10^5 LLC/neo or LLC/IL-1β cells and tumor diameters were measured with calipers in two perpendicular directions every 3 days from day 7.[13] Clodronate liposomes were prepared as described previously.[13,22] On day 20, mice were killed with sodium pentobarbital under deep anesthesia and tumors were harvested for immunohistochemical analysis. Tumor volumes (in mm^3) were calculated as length × width^2 × 0.5. All experimental protocols were approved by the ethics committee of Kyushu University.

Immunohistochemistry. On removal, tumors were snap frozen in OCT compound (Sakura Finetechnical Co. Ltd, Tokyo, Japan) and 5-μm sections were cut, air dried, and fixed in cold acetone for 10 min. The sections were blocked with 3% skim milk for 10 min at room temperature, washed with phosphate-buffered saline and stained with rat antimouse CD31 (BD Biosciences, San Jose, CA, USA) for 20 min at room temperature or rat antimouse F4/80 (Serotec, Raleigh, NC, USA) for 16 h at 4°C, as described previously.[13]

Co-culturing lung cancer cells and macrophages. For the co-culture of lung cancer cells (LLC/neo and LLC/IL-1β) and macrophages (U937), LLC cells at 5×10^5 cells/mL were seeded with or without U937 at 5×10^5 cells/mL in 24-well plates. They were then cultured for 1 day in RPMI with 10% FBS. To determine the protein levels of the angiogenic factors, the cells were further cultured for 48 h in medium with 0.5% FBS, and the medium was collected for ELISA.

Mouse dorsal air sac assay. In the dorsal air sac assay,[13] we used exponentially growing LLC and U937 cells. Chambers containing 1×10^6 LLC/neo or LLC/IL-1β cells with or without 5×10^5 U937 cells were implanted s.c. and the newly formed vessels were determined in the dorsal areas after 5 days.[23]

Human umbilical vein endothelial cell migration assay. LLC/neo, LLC/IL-1β, or U937 cells, or combinations of them, were grown in the outer chamber of Transwell plates (Iwaki, Tokyo, Japan) at 37°C in medium containing 10% FBS.[18] HUVEC were suspended in medium containing 0.5% FBS and 1.5×10^5 cells per inner chamber on polycarbonate filters (8-μm pores; Kurabo, Tokyo, Japan) coated with 1.33 μg/mL human plasma fibronectin (Life Technologies; Gaithersburg, MD, USA). After 8 h at 37°C, the medium was removed from the inner chambers and the cells were removed from the upper surface of the filters with cotton swabs. Cells on the lower surface were fixed with methanol, stained with Giemsa, and counted at a magnification of ×200 in four fields per chamber; the results are presented as the means of four chambers.

Angiogenesis protein array. The production of angiogenesis-related factors was determined using Angiogenesis Antibody Arrays (RayBiotech, Norcross, GA, USA). Integrated density values were measured for each spot and normalized to positive controls on each membrane using Image Gauge version 3.41 (Fuji Photo Film, Tokyo, Japan).

DNA-binding activity of multiple transcription factors. Transcription array was carried out as described previously.[24] TransFactor kits (BD Biosciences, Takara Bio, Otsu, Shiga, Japan) for identifying DNA–protein interactions were used for rapid, high-throughput detection of the activities of mouse transcription factors of NF-κB–p65 and –p50, c-Fos, CREB-1, ATF2, c-Rel, FosB, JunD, SP1, STAT1, and c-Jun in LLC/Neo and LLC-IL-1β nuclear extracts (20 μg). The TransFactor kits were also used to identify the human transcription factor activities of NF-κB–p65 and –p50, c-Fos, CREB-1, ATF2, and c-Rel in human lung B203L nuclear extracts (30 μg). To identify human FosB, JunB, and JunD in nuclear extracts (20 μg), we used Trans AM assay kits (Active Motif, Carlsbad, CA, USA). In this assay, B203L cells were stimulated with or without IL-1β (1 ng/mL) for 6 h in serum-free medium. After the addition of chromogen, plates were read in a plate reader at 650 nm.

Cytokine assays. Exponentially growing cells were cultured for 24 h in 10% FBS, and then for a further 24–48 h with or without IL-1β in serum-free medium. The cytokines present in these culture supernatants were measured by ELISA (R & D Systems). The results are presented as means ± SD.

Gelatin zymography. Secreted metalloproteinase activity was measured by zymography as described previously.[25]

Western blot analysis. Western blot analysis was carried out as described previously.[23,25]

EMSA. EMSA was carried out as described previously.[26] The oligonucleotides used were 5′-GATCAGGGACTTTCCGCTG-GGGACTTTCCAG-3′ and 3′-TCCCTGAAAGGCGACCCCT-GAAAGGTCTCTCGA-5′ for NF-κB, 5′-AATTACCGGGCGGG-CGGGCTACCGGGCGGGT-3′ and 3′-TGGCCCGCCCGCCCG-ATGGCCCGCCCGATGCA-5′ for AP-1, and 5′-AATTACCGGGA-GGGCGGGCTACCGGGAGGGCT-3′ and 3′-TGGCCCTCCC-GCCCGATGGCCCTCCCGATGCA-5′ for Sp1.

Transfection with siRNA. Duplexes of siRNA, corresponding to nucleotide sequences from NF-κB p65 (5′-AUCCGGUGACGA-UCGUCUGUAUCUG-3′) and c-Jun (5′-UUUAAGCUGUGCCACC-UGUUCCCUG-3′), and a negative control siRNA (Invitrogen, Carlsbad, CA, USA), were transfected using Lipofectamine 2000 and Opti-MEN medium (Invitrogen) according to the manufacturer's instructions. Reductions in NF-κB p65 and c-Jun expression were confirmed by western blot analysis.

Statistical analysis. Statistical analysis, using the Mann–Whitney *U*-test and Student's *t*-test, was carried out using JMP 5.01 software (SAS Institute, Cary, NC, USA), with *P*-values below 0.05 considered statistically significant.

Results

Macrophage depletion-induced inhibition of IL-1β-dependent tumor growth and angiogenesis. We previously reported that administration of clodronate liposomes inhibits IL-1β-induced angiogenesis in mouse cornea by reducing macrophage numbers in the blood and cornea.[13] In mice bearing LLC tumors, clodronate liposomes reduced the number of macrophages in the peripheral blood by half to two-thirds of untreated controls (data not shown). We examined the antitumor effects of clodronate liposomes on mice bearing LLC/neo and LLC/IL-1β tumors: the tumor growth rates of LLC/IL-1β were significantly ($P < 0.01$) higher than those of LLC/neo when transplanted to the syngeneic C57BL/6 male mice.[13] Treating mice bearing LLC/neo and LLC/IL-1β tumors with clodronate liposomes reduced the rate of tumor growth, and this effect was greater and more significant for LLC/IL-1β than for LLC/neo tumors (Fig. 1a). Figure 1b shows hematoxylin–eosin stainings of sections of LLC/neo and LLC/IL-1β tumors with or without administration of clodronate liposomes. Immunohistology showed that F4/80-positive macrophages infiltrated both types of tumor, but the numbers were higher in LLC/IL-1β tumors than in LLC/neo tumors (Fig. 1c). Macrophage infiltration into LLC/IL-1β tumors was significantly blocked by clodronate liposomes (Fig. 1d). CD31-positive vascular endothelial cells also demonstrated a markedly higher density of microvessels in LLC/IL-1β tumors compared with LLC/neo tumors, and this was also reduced by clodronate liposomes, markedly in LLC/IL-1β tumors (Fig. 1e,f). Therefore, macrophage infiltration, tumor growth, and angiogenesis were enhanced in LLC/IL-1β tumors compared with LLC/neo tumors, and depletion of the macrophages markedly inhibited both angiogenesis and growth in LLC/IL-1β tumors.

Enhanced angiogenesis by both IL-1β-expressing cancer cells and macrophages. To understand how macrophages affect angiogenesis and tumor growth, we examined vascular endothelial cell migration in cocultures with macrophages. In *in vitro* migration chamber assays, LLC cells were seeded in the outer chambers, with or without macrophages, and vascular endothelial cells were seeded in the inner chambers, separated by polycarbonate filters. When LLC/neo, LLC/IL1β, or U937 cells alone, or U937 with LLC/neo cells together, were present in the outer chamber there was no effect on the migration of endothelial cells,

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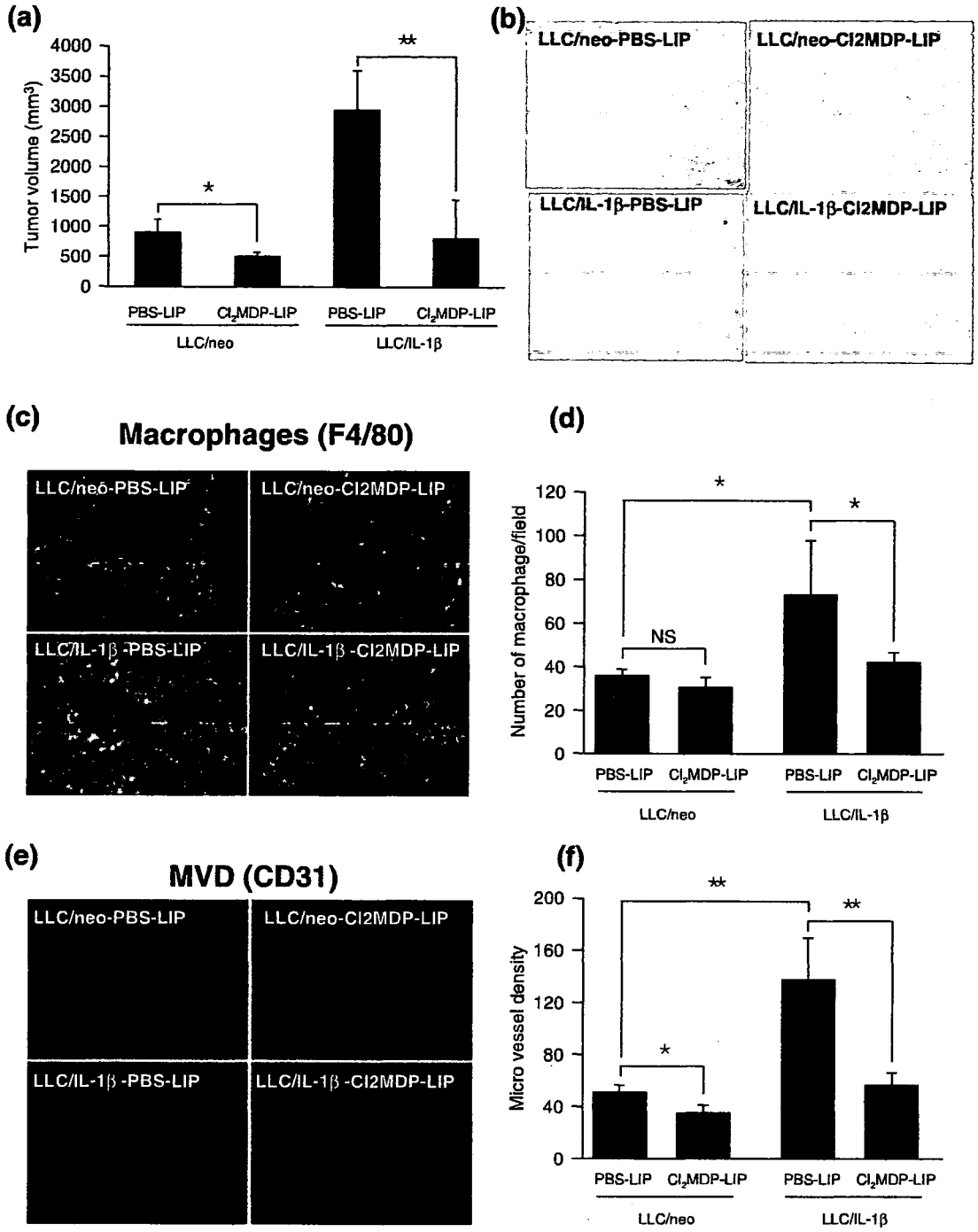

Fig. 1. Effect of clodronate liposomes (Cl_2MDP-LIP) on Lewis lung carcinoma (LLC) tumors *in vivo*. (a) Inhibition of LLC/neo and LLC/IL-1β tumor growth by clodronate liposomes. On day 0, 5×10^5 LLC cells were implanted s.c. into each C57BL/6 mouse. Clodronate-containing or control liposomes containing phosphate-buffered saline (PBS-LIP) were injected every 3 days from day 7. The data represent mean tumor volumes ± SD ($n = 5$ mice). For tumor volumes on day 20, $^*P = 0.012$, $^{**}P < 0.001$. (b) Representative hematoxylin–eosin images of LLC/neo and LLC/IL-1β tumor sections (magnification: ×200). (c) Representative images of LLC tumor sections stained for F4/80 (magnification: ×200). (d) Numbers of infiltrating macrophages in LLC tumors, counted in four microscope fields (magnification: ×200). Data represent means ± SD. $^*P < 0.01$, NS, not significant. (e) Representative images of LLC tumor sections stained for CD31 (magnification: ×200). (f) CD31-positive microvessels quantified by morphometric analysis in LLC tumors in four microscope fields (magnification: ×200). Data represent means ± SD. $^*P < 0.05$, $^{**}P < 0.001$.

compared to medium alone (Fig. 2a). In contrast, there was a significant increase in vascular endothelial cell migration when both U937 and LLC/IL1β cells were present (Fig. 2a). In the mouse dorsal air-sac assay, the presence of U937, LLC/IL-1β, or LLC/IL-1β plus U937 in the chamber promoted the development of capillary networks in the dorsal subcutis in contact with the implanted chamber (Fig. 2b). New development of capillary networks, presumably tumor neovasculatures, with curled structures and tiny bleeding spots was observed in addition to the preexisting microvessels (Fig. 2b), consistent

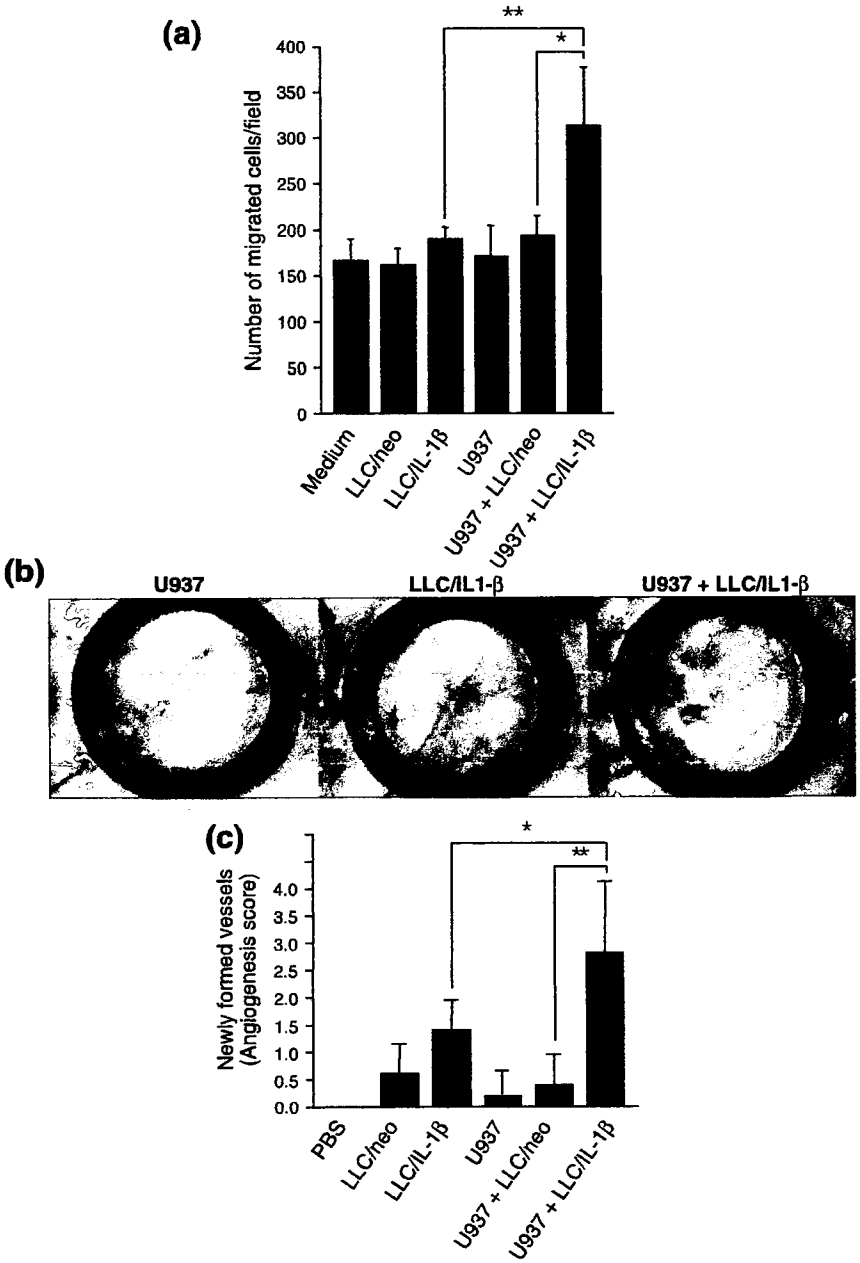

Fig. 2. Angiogenic activity induced by coculturing Lewis lung carcinoma (LLC)/neo or LLC/IL-1β cells with U937 cells. (a) Migration of human umbilical vein endothelial cells (HUVEC) when LLC/neo, LLC/IL-1β, U937, or co-cultures of LLC and U937 cells were grown in the outer chamber for 24 h in 0.5% fetal bovine serum, before seeding 1.5×10^5 HUVEC per well in the inner chamber, on polycarbonate filters precoated with human plasma fibronectin. After 8 h incubation at 37°C, HUVEC that had migrated to the lower surface of the filter were counted in four fields per chamber at a magnification of ×200. Data represent means ± SD ($n = 4$ chambers). $^*P < 0.01$, $^*P < 0.001$. (b) Angiogenesis induced by LLC, U937, and combinations of LLC and U937 cells in the murine dorsal air-sac assay. Chambers containing 1×10^6 LLC or 5×10^5 U937 cells or combinations of LLC and U937 cells were implanted s.c. On day 5 after the implantation, the chambers were removed, and photographs of the implantation sites were taken. (c) Quantitative analysis of newly formed vessels induced by LLC tumors in combination with macrophages on day 5. The newly formed vessels greater than 3 mm in length were counted. Data represent means ± SD ($n = 4$ mice). $^*P < 0.01$, $^{**}P < 0.001$.

with our previous study,[27,28] and we scored and quantified these newly formed vessels. Implanted chambers containing LLC/IL-1β with U937 cells showed higher levels of neovascularization than chambers containing LLC/IL-1β or U937 alone (Fig. 2b). LLC/IL-1β induced approximately two-fold higher levels of neovasculatization than LLC/neo, but there was no statistical difference (Fig. 2c). U937 alone or U937 with LLC/neo cells induced very similar levels of neovascularization compared with LLC/neo cells alone (Fig. 2c). However, co-inoculation of LLC/IL1-β and U937 cells was five-fold more effective in inducing neovascularization compared with both LLC/neo and U937 cells (Fig. 2c).

Enhanced expression of angiogenic factors and chemokines through activation of NF-κB and AP-1 by IL-1β. We first looked at the effect of IL-1β on the production of VEGF-A and MCP-1 in LLC/IL-1β and LLC/neo cells in culture. ELISA confirmed that mouse VEGF-A and MCP-1 levels were significantly higher in LLC/IL-1β compared with LLC/neo cells (Fig. 3a,b). We next looked at the effect of IL-1β on the expression of cytokines and angiogenic factors by human macrophages. IL-8, MCP-1, PDGF-BB, VEGF-A, VEGF-D, and leptin were also increased by IL-1β, and the expression levels of RANTES and PIGF were slightly enhanced (Fig. 3c). Of these factors, the most marked effects were on MCP-1 and IL-8 expression. The expression levels of MCP-1, VEGF-A, and leptin were therefore upregulated by IL-1β in both human U937 and mouse LLC cells. We also compared the expression of human VEGF-A, IL-8, and MCP-1 by U937 cells co-cultured with LLC/neo and LLC/IL-1β cells (Fig. 3d–f). The production of human VEGF-A, IL-8, and MCP-1 was markedly enhanced, by four to five times, only when co-cultured with LLC/IL-1β cells (Fig. 3d–f).

Effect of NF-κB inhibitor on MCP-1, IL-8, VEGF, and MMP-9 induction by IL-1β. Because the effects of IL-1β and other inflammatory cytokines are often mediated through NF-κB, we next asked whether the upregulation of MCP-1, IL-8, and VEGF-A by IL-1β in U937 cells was also mediated through NF-κB. The NF-κB inhibitor DHMEQ at 1 μg/mL significantly blocked the upregulation

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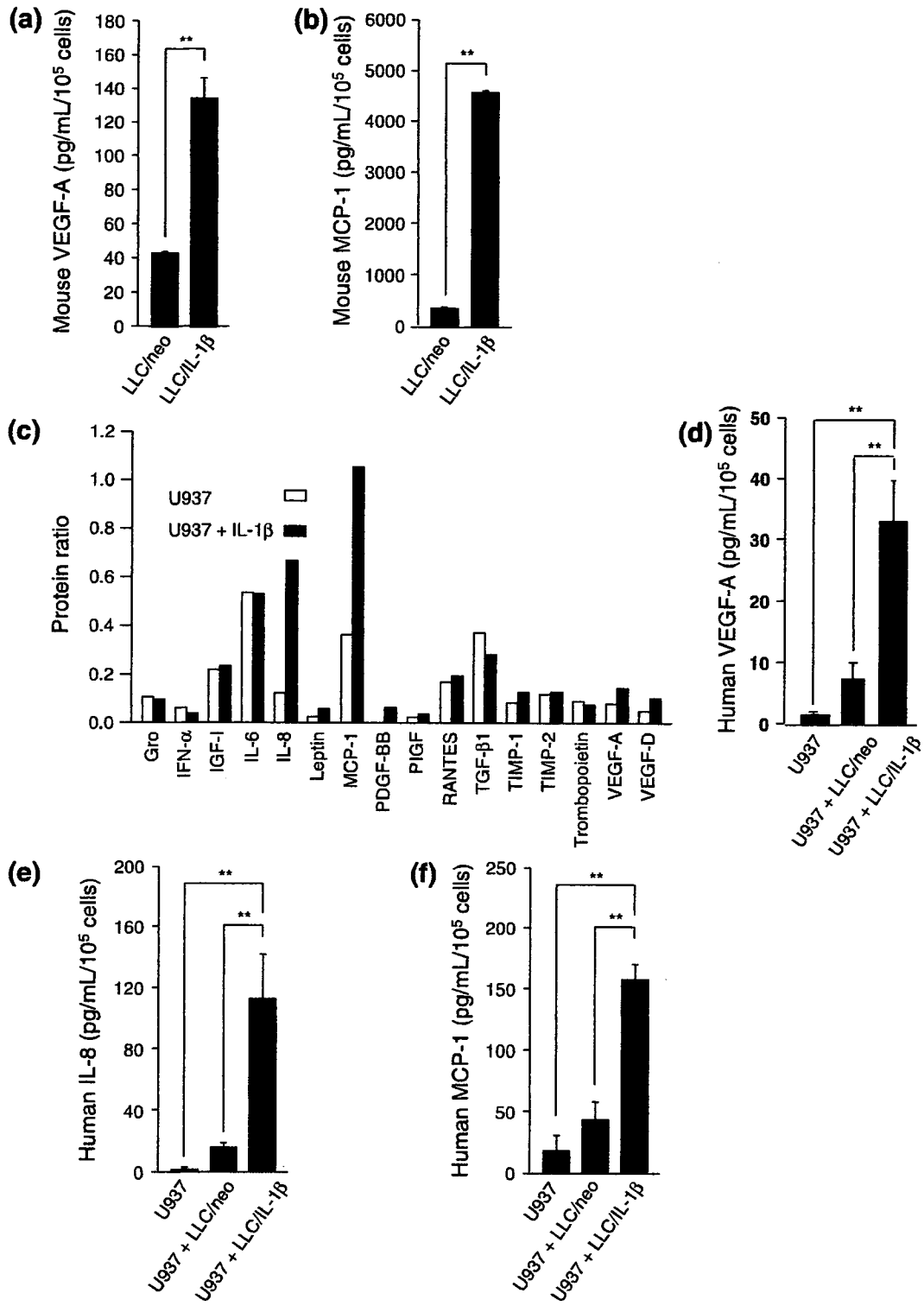

Fig. 3. Expression of angiogenic factors in lung cancer cells and macrophages in culture. Production of (a) vascular endothelial growth factor (VEGF)-A and (b) monocyte chemoattractant protein (MCP)-1 by Lewis lung carcinoma (LLC)/neo and LLC/interleukin (IL)-1β cells was determined with conditioned medium from cell cultures by enzyme-linked immunosorbent assay (ELISA). $^{**}P < 0.01$ ($n = 3$). (c) Production of angiogenic factors was measured using angiogenesis antibody arrays in conditioned medium from U937 cells cultured with or without 1 ng/mL IL-1β. Human (d) VEGF-A, (e) IL-8, (f) and MCP-1 expression measured by ELISA from LLC/neo, LLC/IL-1β, and U937 cells, and co-cultures of LLC and U937 cells, collected after 48 h in medium with 0.5% fetal bovine serum. Data represent means ± SD ($n = 4$). NS, not significant, $^{*}P < 0.05$, $^{**}P < 0.001$.

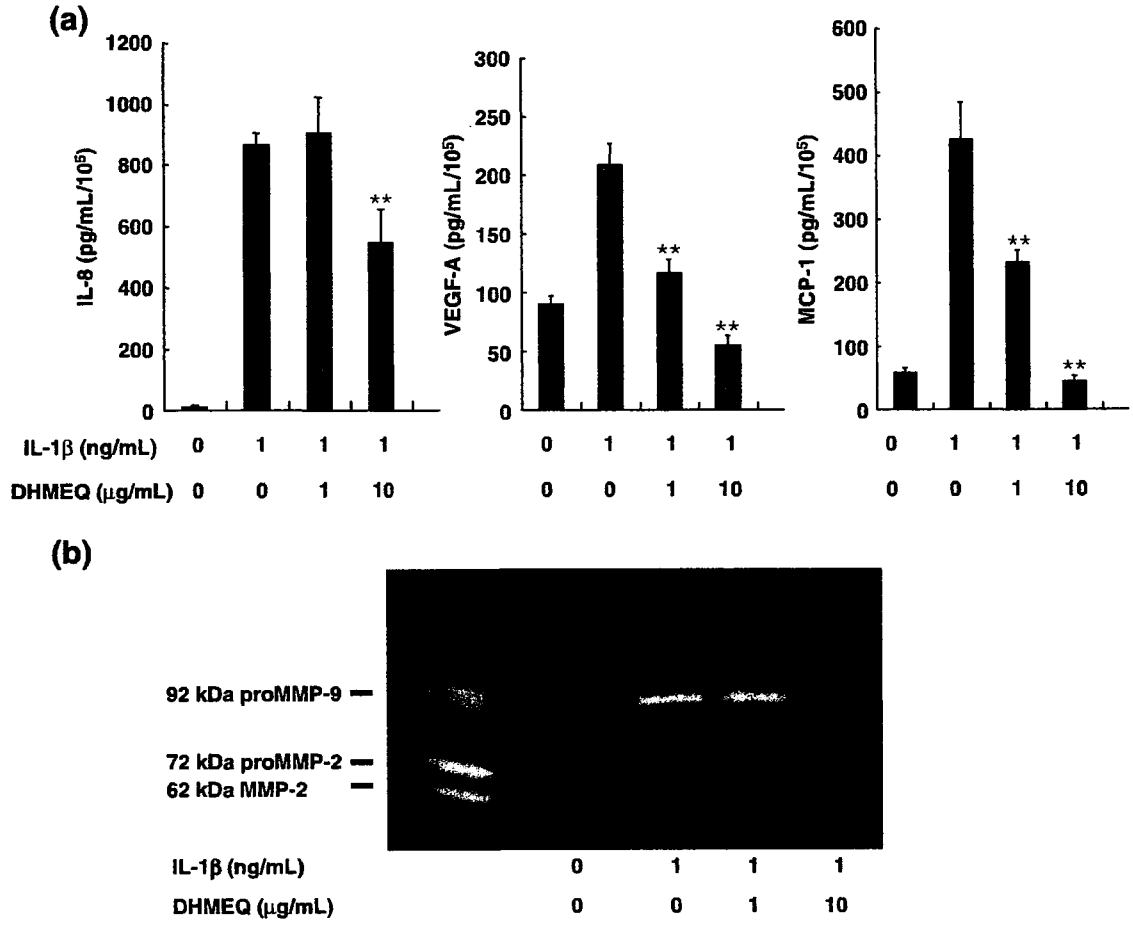

Fig. 4. Effects of a nuclear factor-κB inhibitor on interleukin (IL)-1β-induced monocyte chemoattractant protein (MCP)-1, vascular endothelial growth factor (VEGF)-A, IL-8, matrix metalloproteinase (MMP)-2, and MMP-9 production by U937 cells. (a) ELISA was used to measure MCP-1, IL-8, and VEGF-A production by IL-1β-treated U937 cells in the absence or presence of the NF-κB inhibitor dehydroxymethylepoxiquinomicin. Data represent means ± SD ($n = 4$). $^{**}P < 0.01$, $^{*}P < 0.05$. (b) Gelatin zymography showing three bands with MMP activity corresponding to 92 (proMMP-9), 72 (proMMP-2), and 62 kDa (MMP-2).

of both MCP-1 and VEGF-A by IL-1β, and at 10 μg/mL inhibited IL-8 upregulation by approximately 30% (Fig. 4a). Gelatin zymography showed increased MMP-9, but not MMP-2, activity by IL-1β, and this increased activity was also markedly inhibited by NF-κB inhibitor (Fig. 4b).

Activation of NF-κB and AP-1 in lung cancer cells by IL-1β. We asked which transcription factors were specifically involved in the induction of MCP-1, IL-8, and VEGF-A by IL-1β. We observed higher expression of p65 and p50 (NF-κB), JunD and c-Jun in LLC/IL-1β cells compared with LLC/neo cells, but no difference in the expression of CREB-1 and ATF-1 between the two lines (Fig. 5a). A more than two-fold increase in NF-κB (p50), CREB-1, and ATF-2 was observed in B203L cells by IL-1β (Fig. 5b). NF-κB (p50) was thus activated in both mouse and human lung cancer cells whereas AP-1 (Jun/Fos) was activated only in mouse cells.

The binding of NF-κB to its consensus sequence was analyzed in nuclear extracts of U937 and lung cancer cells by EMSA. The activity of NF-κB binding to its consensus motif was enhanced after 6 h stimulation with IL-1β in macrophages, whereas this enhancement was seen after only 15 min stimulation in two human lung cancer cell lines (A549 and B203L) (Fig. 5c). Of the two major DNA–protein complexes formed, the upper band corresponded to the NF-κB–DNA complex, as this was activated specifically by IL-1β and was also reduced in a dose-dependent manner by adding exogenous double-stranded NF-κB oligonucleotides.

Decreased expression of IL-1β induced upregulation of IL-8, VEGF-A, and MCP-1 by knockdown of NF-κB and AP-1. We next examined the effect of knockdown of NF-κB (p65) and c-Jun on the enhanced expression of IL-8, VEGF-A, and MCP-1 by IL-1β. Because transfecting macrophages by growing them in suspension with siRNA has a low efficiency, monolayers of two human lung cancer cell lines, A549 and B203L, were used as recipients. Transfection with p65 siRNA almost completely knocked down p65 protein expression in the total cell fraction and transfection with c-Jun siRNA similarly knocked down c-Jun protein in the nuclear fraction (Fig. 6a). The IL-1β-induced upregulation of VEGF-A and IL-8 was significantly inhibited by p65 siRNA and c-Jun siRNA (Fig. 6b). In contrast, the IL-1β-induced upregulation of MCP-1 was significantly inhibited by only p65 siRNA, but not by c-Jun siRNA (Fig. 6b). AP-1 as well as NF-κB might play important roles in the induction of two angiogenic factors, VEGF-A and IL-8, and NF-κB might be specifically involved in the induction of VEGF-A, IL-8, and MCP-1.

Discussion

Interleukin-1β is known to be overexpressed in various malignancies,[29,30] and to be essential for inflammatory responses

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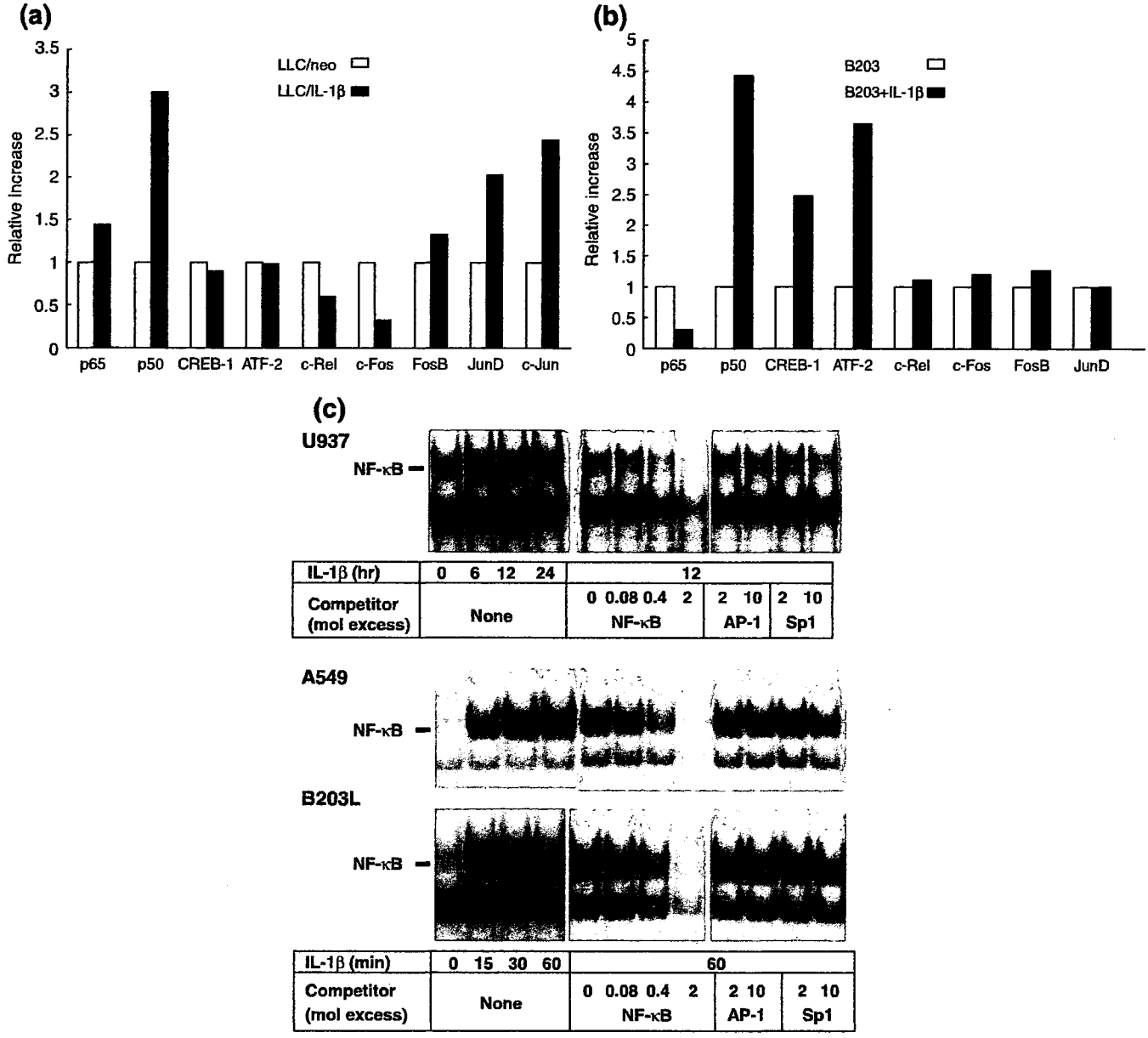

Fig. 5. Transcription factors in interleukin (IL)-1β-stimulated mouse and human lung cancer cells, and nuclear factor (NF)-κB activation by IL-1β in human macrophages and lung cancer cells. (a) Expression profiles of transcription factors expressed by mouse Lewis lung carcinoma (LLC)/neo and LLC/IL-1β cells using transcription arrays. A relative increase in transcription factors in LLC/IL-1β cells was seen when normalized to LLC/neo cells. (b) Expression profiles of transcription factors expressed by human lung B203L cells in the absence or presence of 1 ng/mL IL-1β. A relative increase in transcription factors in IL-1β-treated cells was seen when normalized to untreated cells. (c) Effect of IL-1β on binding to the NF-κB consensus fragment in human U937 cells and the human lung cancer cell lines A549 and B203L. After incubation for the times indicated with 1 ng/mL IL-1β, nuclear extracts were incubated with ^{32}P-labeled NF-κB consensus fragment and subjected to gel electrophoresis with increasing molar excesses of unlabeled AP-1, Sp1, or NF-κB DNA fragments. The position of the retarded NF-κB protein complex is indicated on the left.

and tumor growth in various animal models.[31–33] In IL-1β-knockout mice, reduced tumor growth and angiogenesis were reported.[33,34] Zeisberger *et al.* have reported that administration of clodronate encapsulated in liposomes depletes TAM in mouse models, resulting in significant inhibition of tumor growth and angiogenesis, whereas clodronate alone does not.[35] Consistent with this study, our present study also demonstrated that clodronate encapsulated in liposomes blocks both LLC/IL-1β tumor growth and angiogenesis, specifically by eliminating macrophages. In both the dorsal air-sac model *in vivo* and endothelial cell-migration assays *in vitro*, we have shown that LLC/IL-1β and U937 cells synergistically induce angiogenesis compared with either cell line alone. This supports the idea that macrophages together with cancer cells provide a microenvironment favorable to endothelial-cell migration and also angiogenesis.

Various angiogenic factors, including VEGF-A and IL-8, are upregulated by IL-1β in cancer cells,[32] and blocking IL-1 signaling can markedly curtail tumor growth.[36] Expression of MCP-1 and VEGF-A was markedly upregulated in LLC/IL-1β cells, and IL-1β stimulated not only the production of VEGF-A and IL-8, but also that of VEGF-A, VEGF-D, PDGF-BB, PIGF, and MCP-1 in macrophages. These angiogenesis-related factors are expected

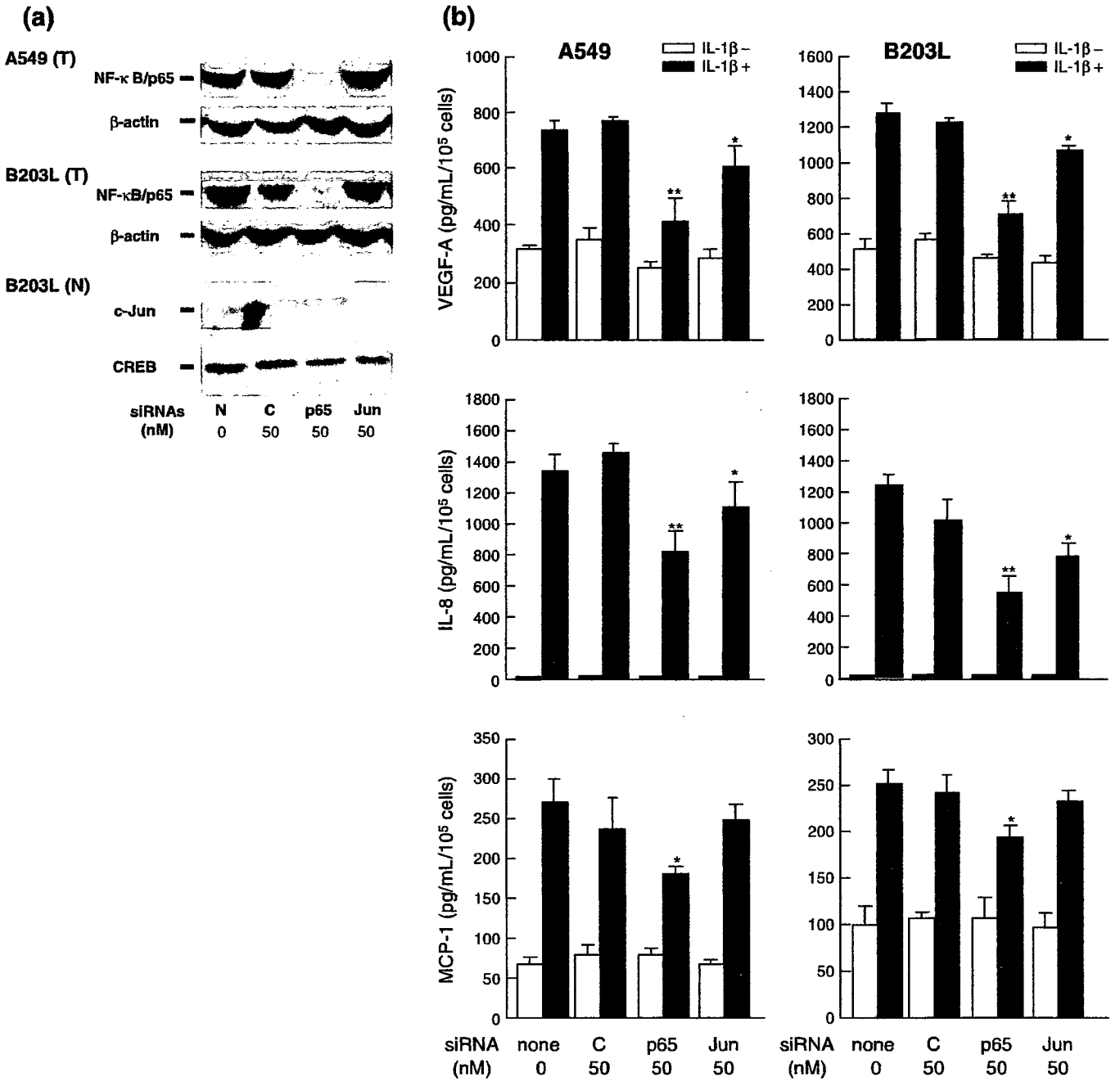

Fig. 6. Effect of nuclear factor (NF)-κB/p65 and AP-1/c-Jun small interference RNA (siRNA) on interleukin (IL)-1β-induced IL-8 expression of vascular endothelial growth factor (VEGF)-A, IL-8, and monocyte chemoattractant protein (MCP)-1. (a) Two human lung cancer cell lines, A549 and B203L, were treated with 50 nM NF-κB/p65 siRNA, 50 nM AP-1/Jun siRNA, or 50 nM control siRNA for 48 h. Total cellular lysates (for NF-κB) or nuclear fractions (for c-Jun) were analyzed by western blot analysis. (b) After treatment with siRNA, cells were incubated for a further 24 h with IL-1β. Conditioned medium was then collected, and VEGF-A, IL-8, and MCP-1 were measured by ELISA. Data are means ± SD ($n = 3$). $^{**}P < 0.001$ and $^{*}P < 0.05$, compared with control siRNA. None, lipofectamine alone; C, control siRNA; Jun, c-Jun siRNA.

to promote further tumor angiogenesis in response to inflammatory stimuli. We also observed enhanced MMP-9 activity by IL-1β in macrophages. The expression of MMP-9 is reported to be markedly enhanced in monocytes and macrophages by several CXC chemokines, promoting the migration and proliferation of both vascular endothelial cells and cancer cells.[37,38] MMP-9, as well as other angiogenic factors expressed by macrophages and cancer cells, thus appears to play a critical role in tumor growth and angiogenesis.

Tumor-associated macrophages are often recruited into tumors by MCP-1, which is also closely associated with inflammatory angiogenesis,[39,40] both in cancer and the cornea,[13,41,42] and by CCL5 (RANTES), which stimulates the expression of MCP-1, CCL3 (MIP-1α), CCL4 (MIP-1β), and IL-8 from monocytes.[38] However, under the experimental conditions used, we observed a marked increase in MCP-1 and IL-8 production, but only a slight increase in RANTES production by macrophages in response to IL-1β. MCP-1 production was particularly enhanced

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by IL-1β in cancer cells and macrophages respectively, and MCP-1 production was synergistically enhanced in co-culture. MCP-1 is a chemoattractant known to be essential for the recruitment of angiogenic macrophages.[2,43] We have reported that in MCP-1-knockout mice, the IL-1β-induced infiltration of macrophages into corneas is impaired,[2,13] confirming a pivotal role for MCP-1 in the recruitment and activation of macrophages.

Nuclear factor-κB is a transcription factor that is known to mediate the effects of proinflammatory cytokines and is an obvious candidate for mediating the effects of IL-1β on angiogenic factors, chemoattractants, and COX2. NF-κB activation promotes cancer progression in various experimental animal models,[44,45] and its specific inactivation can attenuate tumor progression.[46] We previously demonstrated a role for both NF-κB and AP-1 in the cytokine-induced upregulation of VEGF-A and IL-8.[11,12,17] In the present study, we have further suggested that the enhanced expression of IL-8 and VEGF-A induced by inflammatory stimuli is highly susceptible to interference by NF-κB and AP-1 siRNA. Moreover, IL-8 promoter activation was shown to be dependent on NF-κB and AP-1, but to a lesser extent on NF-IL-6.[16] The production of VEGF-A, IL-8, and MCP-1 in IL-1β-treated cells was also blocked by NF-κB inhibitor. Taken together, the transcription factors NF-κB and AP-1 might be mainly involved in the inflammatory cytokine-induced upregulation of potent angiogenic factors and chemokines.

Of the many chemokines, MCP-1 is a potent chemoattractant for macrophages and is expected to further promote monocyte and macrophage recruitment into tumors. Activated TAM in the tumor stroma could promote angiogenesis and the proteinase-dependent migration of cancer cells and vascular endothelial cells, thus setting up a cycle of proliferation, invasion, metastasis, and angiogenesis. Activated TAM can therefore be highly angiogenic, through interaction with cancer cells, and create a tumor microenvironment favorable for the acquisition of malignant characteristics by cancer cells. Macrophages are therefore critical in the development of blood vessels, under the influence of inflammatory stimuli in the tumor stroma, which can switch cancer cells to progress to malignancy. Novel therapeutic strategies for cancer should therefore be developed by targeting inflammatory angiogenic processes in the tumor stroma.

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Expression of HER2 and Estrogen Receptor α Depends upon Nuclear Localization of Y-Box Binding Protein-1 in Human Breast Cancers

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Abstract

In our present study, we examined whether nuclear localization of Y-box binding protein-1 (YB-1) is associated with the expression of epidermal growth factor receptors (EGFR), hormone receptors, and other molecules affecting breast cancer prognosis. The expression of nuclear YB-1, clinicopathologic findings, and molecular markers [EGFR, HER2, estrogen receptor (ER)α, ERβ, progesterone receptor, chemokine (C-X-C motif) receptor 4 (CXCR4), phosphorylated Akt, and major vault protein/lung resistance protein] were immunohistochemically analyzed. The association of the expression of nuclear YB-1 and the molecular markers was examined in breast cancer cell lines using microarrays, quantitative real-time PCR, and Western blot analyses. Knockdown of YB-1 with siRNA significantly reduced EGFR, HER2, and ERα expression in ERα-positive, but not ERα-negative, breast cancer cell lines. Nuclear YB-1 expression was positively correlated with HER2 (P = 0.0153) and negatively correlated with ERα (P = 0.0122) and CXCR4 (P = 0.0166) in human breast cancer clinical specimens but was not correlated with EGFR expression. Nuclear YB-1 expression was an independent prognostic factor for overall (P = 0.0139) and progression-free (P = 0.0280) survival. In conclusion, nuclear YB-1 expression might be essential for the acquisition of malignant characteristics via HER2-Akt–dependent pathways in breast cancer patients. The nuclear localization of YB-1 could be an important therapeutic target against not only multidrug resistance but also tumor growth dependent on HER2 and ERα. [Cancer Res 2008;68(5):1504–12]

Introduction

Nuclear localization of Y-box binding protein-1 (YB-1) is required for its transcriptional control of multidrug resistance–related genes and for its action in repairing DNA damage induced by anticancer agents and radiation in cancer cells; as a result of these actions, it is responsible for the acquisition of global drug resistance to a wide range of anticancer agents (1, 2). Immunohistochemical analyses have shown that nuclear YB-1 localization is a target marker of intrinsic importance for global drug resistance in cancer (2). Bargou et al. (3) reported that nuclear localization of YB-1 was associated with P-glycoprotein expression in human primary breast cancers, and other immunohistochemical studies have shown an association between YB-1 and P-glycoprotein in osteosarcoma, synovial sarcoma, breast cancer, ovarian cancer, and prostate cancer (4–12). Fujita et al. (13) reported that the increase in P-glycoprotein expression when patients were treated with paclitaxel was accompanied by nuclear YB-1 localization in breast cancers.

Nuclear expression of YB-1 is often associated with poor prognosis in various human malignancies, including breast cancer (3, 6), ovarian cancer (8, 11), synovial sarcoma (5), and lung cancer (14). In a study using molecular profiling, Faury et al. (15) recently showed that overexpression of YB-1 is a novel prognostic target for pediatric glioblastoma; however, the intracellular localization of YB-1 was not determined. These clinical studies suggest the close involvement of YB-1 in the acquisition of global drug resistance (2); however, it remains unclear whether the association of YB-1 with poor prognosis is due to this effect, as YB-1 nuclear localization is also a prognostic marker irrespective of P-glycoprotein expression (8, 14, 16). This suggests that other factors affecting tumor growth, invasion, and metastasis could also be involved in the association of YB-1 with poor prognosis in malignant cancers (8, 14).

YB-1 gene induced the development of breast cancers of many histologic types in an experimental animal model (17), suggesting that YB-1 is oncogenic (18). YB-1 overexpression in human mammary epithelial cells induced epidermal growth factor (EGF)-independent growth by activating the EGF receptor (EGFR) pathway (18). Jurchott et al. (19) reported that nuclear localization of YB-1 was induced during G_1-S phase transition, accompanied by increased expression of cyclin A and B. These studies suggest a close link between YB-1 expression and the growth potential of breast cancer cells, which might contribute to poor prognosis. Wu and colleagues (20) established a close correlation between YB-1 expression and the expression of EGFR and HER2 in breast cancer patients (n = 389) using tumor tissue arrays. Knock-out of YB-1 in mice caused some embryonic lethality, severe growth retardation, and exencephaly (21, 22). Moreover, fibroblasts derived from YB-$1^{-/-}$ knockout embryos had slower growth rates than those from wild-type embryos, and failed to undergo morphologic transformation *in vitro* (22, 23). Sutherland et al. (24) have also shown that breast cancer cells with defective nuclear localization of YB-1 multiply slowly in monolayers and during anchorage-independent growth.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Taken together, these findings suggest that YB-1 plays a key role in the expression of not only drug resistance–related genes but also cell growth–related genes.

In the present study, we determined whether nuclear YB-1 localization influenced the expression of growth factor and hormone receptors, EGFR, HER2, estrogen receptor (ER)α, and ERβ, in human breast cancers. In addition, we used molecular profiling to examine whether nuclear YB-1 localization affected the expressions of major vault protein/lung resistance protein (MVP/LRP), phosphorylated Akt (p-Akt), progesterone receptor (PgR), and chemokine (C_X_C motif) receptor 4 (CXCR4).

Materials and Methods

Cell lines, protein extraction, and immunoblotting. Human breast cancer cell lines, T-47D, MCF-7, KPL-1, MDA-MB231, and SKBR-3 were obtained from the American Type Culture Collection and were grown as described elsewhere (25). Anti–YB-1 was generated as described previously (26). Anti-EGFR and anti-PTEN antibodies were obtained from Cell Signaling Technology. Anti-HER2 was purchased from Upstate, Inc. Anti-ERα was obtained from Santa Cruz Biotechnology, Inc. Anti-CXCR4 was obtained from Abcam plc. Anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Trevigen, Inc. Anti-MVP/LRP was a kind gift from Professor S. Akiyama (Kagoshima University, Kagoshima, Japan). LY294002 was obtained from Sigma Co. Trastuzumab was purchased from Chugai Pharmaceutical Company. Cells were lysed in cold protein extraction reagent (M-PER; Pierce) with protease inhibitors and phosphatase inhibitors. Nuclear and cytoplasmic fractions were prepared as described previously (27). Lysates were subjected to SDS-PAGE and blotted onto Immobilon membrane (Millipore Corp.). After transfer, the membrane was incubated with the primary antibody and visualized with secondary antibody coupled to horseradish peroxidase and Supersignal West Pico Chemiluminescent Substrate (Pierce). Bands on Western blots were analyzed densitometrically using Scion Image software (version 4.0.2; Scion Corp.).

Microarray analysis. The small interfering RNA (siRNA) corresponding to nucleotide sequences of YB-1 (5′-GGU UCC CAC CUU ACU ACA U-3′) was purchased from QIAGEN Inc. SiRNA duplexes were transfected using Lipofectamine and Opti-MEM medium (Invitrogen) according to the manufacturer's recommendations. Duplicate samples were prepared for microarray hybridization. Forty-eight hours after siRNA transfection, total RNA was extracted from cell cultures using ISOGEN (Nippon Gene Co. Ltd.). Two micrograms of total RNA were reverse transcribed using GeneChip 3′-Amplification Reagents One-Cycle cDNA Synthesis kit (Affymetrix, Inc.) and then labeled with Cy5 or Cy3. The labeled cRNA was applied to the oligonucleotide microarray (Human Genome U133 Plus 2.0 Array; Affymetrix). The microarray was scanned on a GeneChip Scanner3000, and the image was analyzed using a GeneChip Operating Software ver1.

Quantitative real-time PCR. RNA was reverse transcribed from random hexamers using avian myeloblastosis virus reverse transcriptase (Promega). Real-time quantitative PCR was performed using the Real-time PCR system 7300 (Applied Biosystems). In brief, the PCR amplification reaction mixtures (20 μL) contained cDNA, primer pairs, the dual-labeled fluorogenic probe, and Taq Man Universal PCR Master Mix (Applied Biosystems). The thermal cycle conditions included maintaining the reactions at 50°C for 2 min and at 95°C for 10 min, and then alternating for 40 cycles between 95°C for 15 s and 60°C for 1 min. The primer pairs and probe were obtained from Applied Biosystems. The relative gene expression for each sample was determined using the formula $2^{(-\delta Ct)} = 2^{(Ct\ (GAPDH)-Ct\ (target))}$, which reflected the target gene expression normalized to GAPDH levels.

Immunohistochemistry. Anti-EGFR, anti-HER2, anti-ERα, and anti-PgR were obtained from Ventana Medical Systems. Anti-CXCR4 was purchased from Prosci, Inc. Anti-MVP/LRP was obtained from Chemicon. Tissue sections were taken from 73 breast cancer patients who underwent radical surgery in the Department of Surgery, Kurume University Hospital, Japan, between 1993 and 1999. The 4-μm tissue sections were deparaffinized, and the slides were heated in a Cell Conditioning Solution buffer for 60 min at 100°C. The sections were stained using the BenchMark XT (IHC Automated System) and ChemMate ENVISION method (Dako Corporation). BenchMark XT was used for staining anti–YB-1, anti-ERα, anti-EGFR, anti-HER2, and anti-PgR. The ChemMate ENVISION method was used for immunochemical staining of anti-ERβ, anti–p-Akt, anti-CXCR4, and anti-MVP/LRP. The samples were viewed using an Olympus BX51 fluorescence microscope (Olympus). The extent of staining of YB-1, ERα, ERβ, and PgR proteins was classified based on the percentage of cells with strongly stained nuclei: ≥10% indicated that a gland was positive for YB-1, and ≤9% indicated that it was negative. EGFR and HER2 expressions were classified into four categories: score 0, no staining at all or membrane staining in <10% of tumor cells; score 1+, faint/barely perceptible partial membrane staining in >10% of tumor cells; score 2+, weak to moderate staining of the entire membrane in >10% of tumor cells; and score 3+, strong staining of the entire membrane in >10% of tumor cells. The extent of immunohistologic staining for EGFR was defined as follows: scores of 2+ or 3+ were regarded as positive, and scores of 0 or 1+ were regarded as negative. The extent of immunohistologic staining for HER2 was defined as follows: scores of 3+ were regarded as positive, and scores of 0 or 1+ or 2+ were regarded as negative. Immunohistochemical staining of p-Akt and CXCR4 was defined based on the percentage of cells with strong cytoplasmic staining as follows: ≥10% indicated that a gland was positive, whereas ≤9% indicated that it was negative. MVP/LRP staining was defined as follows: ≥50% of cells with a strongly stained cytoplasm indicated that a gland was positive, whereas ≤49% indicated that it was negative. All immunohistochemical studies were evaluated by two experienced observers who were blind to the conditions of the patients.

Statistical analysis. The associations between YB-1 and clinicopathologic findings (age, tumor size, menopausal status, histologic grade, and lymph node metastasis) and molecular markers were tested by Fisher's exact test, the χ^2 test, or the Wilcoxon rank-sum test, depending on the type of data. A P value of <0.05 was regarded as significant unless otherwise indicated. The relationships between YB-1 expression and overall survival/progression-free survival, as well as other clinicopathologic findings and molecular markers, were examined by the Kaplan-Meier method and the log-rank test. Hazard ratios (HR) were estimated by Cox regressions.

As YB-1 and the expression of receptors of the EGFR family and hormone receptors, as well as the clinicopathologic findings, were all correlated, we summarized them by means of their principal components and investigated the relationship between these components and overall survival/progression-free survival by Cox regression. The relationship between the principal components was found to be related to overall survival/progression-free survival, and the clinicopathologic findings and molecular markers were investigated by studying their correlations. In addition, to obtain a direct representation of the relationship between molecular markers, we used a graphical modeling technique incorporating logistic regressions; a path was drawn between two markers only if these markers were conditionally associated with a significance level of 0.1, given the other markers. The data on overall survival and progression-free survival in this analysis were updated on February 27, 2007.

Results

The knock-down of YB-1 alters the expression of EGFR, HER2, ERα, CXCR4, and MVP/LRP genes. We initially compared the expressions of YB-1 siRNA–treated and control MCF-7 breast cancer cells using a high-density oligonucleotide microarray. Of the 54,675 RNA transcripts and variants in the microarray, we identified differentially expressed genes containing 43 genes that were up-regulated >2-fold and 203 genes that were down-regulated 0.5-fold or less (Supplementary Table S1). It has been reported that the activity of PI3K/Akt was required for translocation of YB-1 into the nucleus (24, 27). We therefore investigated the effect of LY294002, a selective inhibitor of PI3K, in both T-47D and MDA-MB231 cells. LY294002 inhibited the

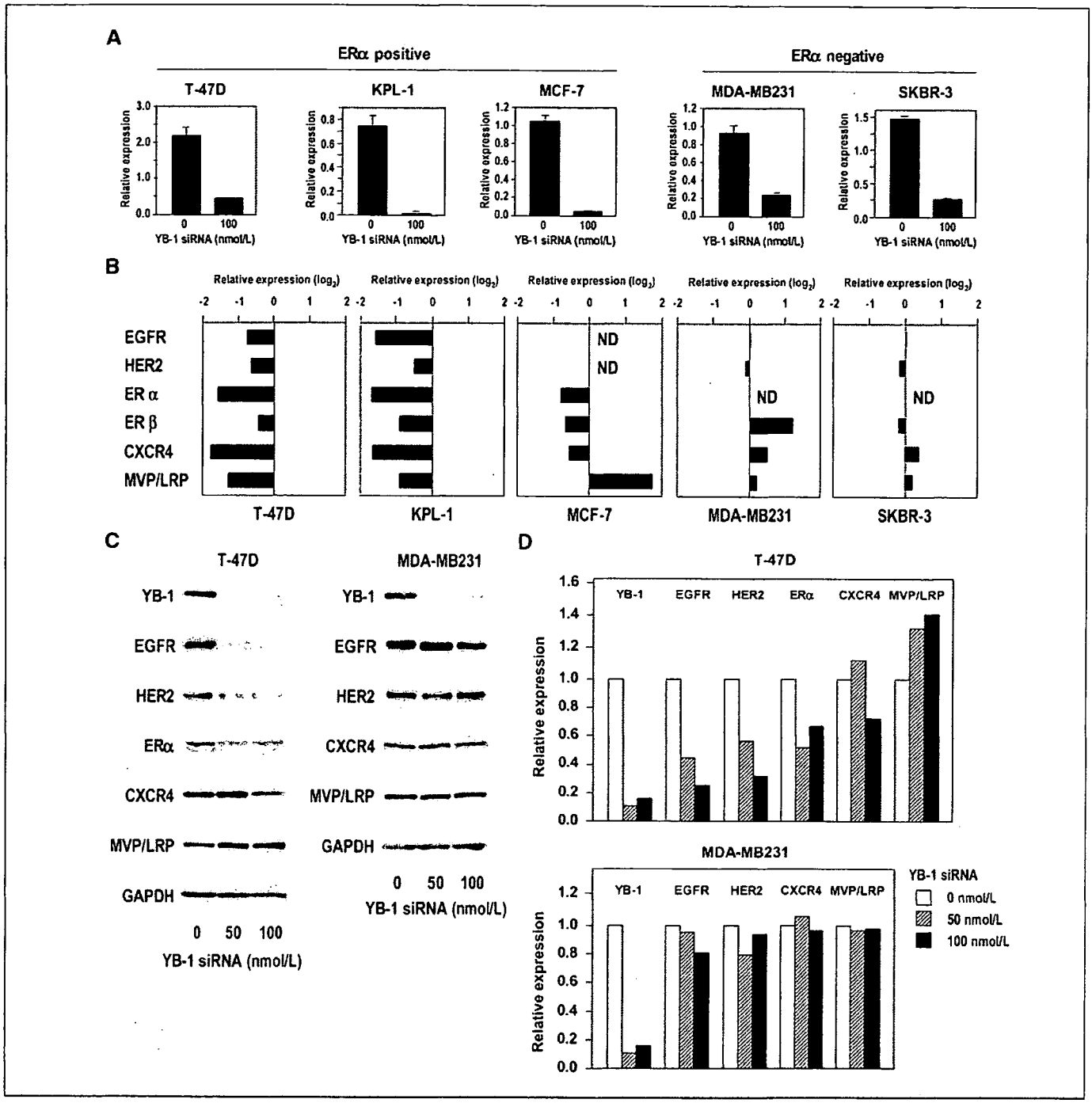

Figure 1. Effect of YB knock-down on expression of EGFR, HER2, ERα, CXCR4, and MVP/LRP in ERα-positive and ERα-negative breast cancer cells. *A*, YB-1 knock-down by treatment of YB-1 siRNA for 48 h. Relative mRNA expression was measured by qRT-PCR. *Columns*, mean of three independent experiments; *bars*, SD. *B*, levels of EGFR, HER2, ERα, ERβ, PgR, CXCR4, and MVP/LRP mRNA expression in YB-1 siRNA–treated cells. Changes of mRNA expression were expressed as the log of relative expression. *ND*, not detected. *C*, T-47D and MDA-MB231 cells were incubated with YB-1 siRNA for 72 h, and lysates were prepared. *D*, levels of YB-1, EGFR, HER2, ERα, CXCR4, and MVP/LRP expression were measured by densitometry.

nuclear expression of YB-1 in both cell lines (Supplementary Fig. S1*A*), consistent with previous reports (24, 27). We also examined whether PTEN status was correlated with nuclear YB-1 expression in breast cancer cells. Of the five human breast cancer cell lines used, cellular levels of the PTEN were not significantly correlated with nuclear expression levels of YB-1 protein (Supplementary Fig. S1*B* and *C*).

The differentially expressed genes included MVP/LRP and CXCR4, consistent with our previous study on human ovarian cancer cells (27). We next tested, by quantitative real-time PCR (qRT-PCR), whether the expression of CXCR4 and MVP/LRP was affected by knock-down of YB-1 in various human breast cancer cell lines. We also examined the expressions of growth factor receptors and hormone receptors, such as EGFR, HER2, ERα, and

ERβ, which are thought to be important target genes in breast cancer. The five cell lines used were as follows: T-47D, MCF-7, and KPL-1, which are ERα-positive; and MDA-MB231 and SKBR-3, which are ERα-negative. Transfection of YB-1 siRNA decreased the expression of YB-1 mRNA by ≥70% in all five cell lines (Fig. 1*A*). Both EGFR and HER2 mRNA levels were found to be decreased in YB-1 siRNA–treated T-47D and KPL-1 cells but not in MDA-MB231 and SKBR-3 cells (Fig. 1*B*). EGFR and HER2 mRNAs were not detected in MCF-7 cells. It has been reported that the 5′ regulatory region of the ERα gene contains several Y-box–like sequences. Cellular mRNA levels of ERα were reduced by YB-1 siRNA in T-47D, KPL-1, and MCF-7 cells by 74%, 75%, and 40%, respectively, (Fig. 1*B*). CXCR4 and MVP/LRP mRNA levels were also decreased in YB-1 siRNA–treated T-47D and KPL-1 cells but not in MDA-MB231 and SKBR-3 cells (Fig. 1*B*).

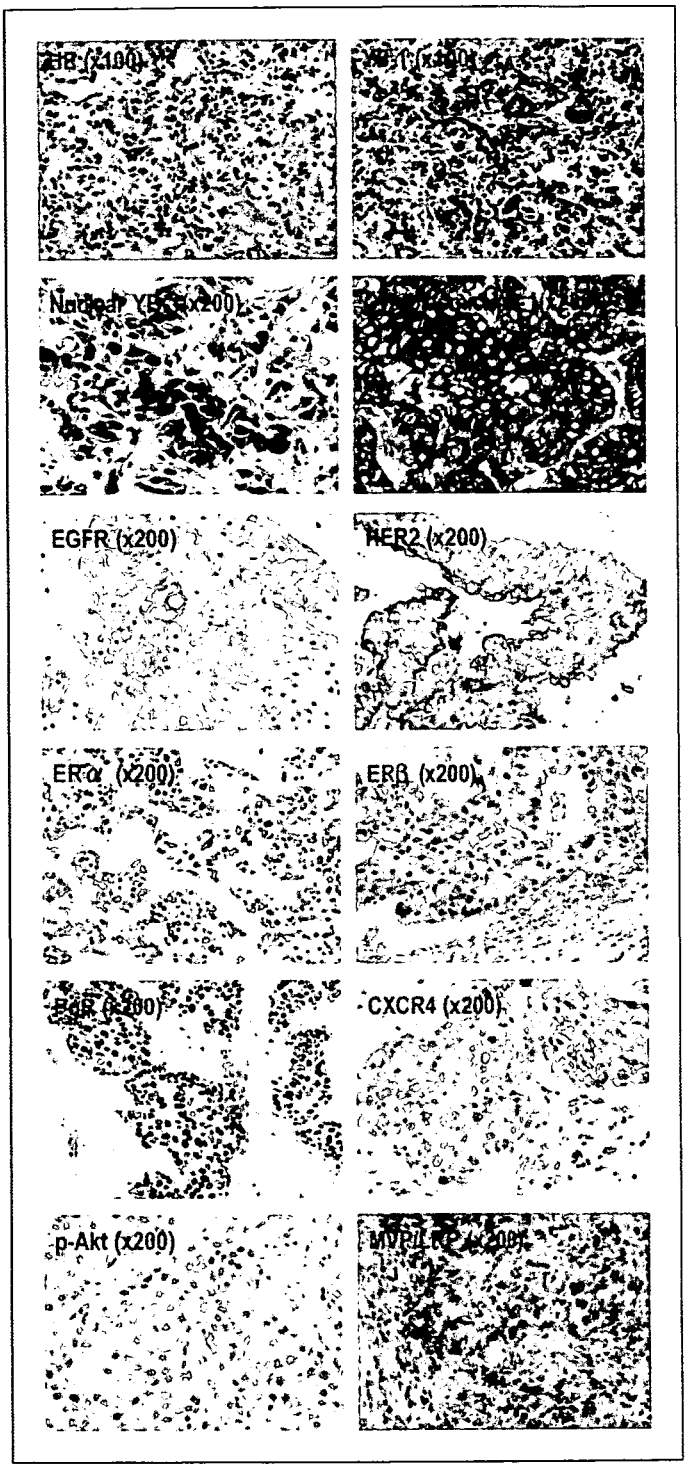

Figure 2. Histologic findings and expression of YB-1, EGFR, HER2, ERα, ERβ, PgR, CXCR4, p-Akt, and MVP/LRP in human breast cancer. YB-1 expression was recognized in two patterns: nuclear positive or negative. Cancer cells showed strong expression of EGFR and HER2 in the membrane. Strong expression of ERα, ERβ, and PgR was found in the nucleus. Moderate-to-strong expressions of CXCR4, p-Akt, and MVP/LRP were found in the cytoplasm.

Western blot analysis showed that siRNA to YB-1 decreased protein levels of EGFR, HER2, and ERα in T-47D cells; however, we did not observe decreased expression of EGFR and HER2 as a result of YB-1 knock-down in MDA-MB231 cells (Fig. 1*C* and *D*). These observations show that YB-1 only reduces the expression of EGFR and HER2 when ERα is present. Moreover, the expression of ERα was also affected by YB-1 knock-down. We next examined the causal relationship between HER2, ERα, and nuclear YB-1 in breast cancer cells in culture. Treatment with HER2-targeting trastuzumab blocked the nuclear localization of YB-1 in both T-47D and MDA-MB231, but the inhibitory effect was less in MDA-MB231 (Supplementary Fig. S2*A*). By contrast, nuclear YB-1 expression was not affected by ERα knock-down in T-47D cells in culture (Supplementary Fig. S2*B*). HER2 might directly modulate the cellular localization of YB-1 in breast cancer cells; however, ERα might not directly affect nuclear YB-1 localization.

Immunostaining of EGFR, HER2, ERα, ERβ, CXCR4, p-Akt, and MVP/LRP in human breast cancers. To examine which genes are specifically associated with nuclear YB-1 localization in human breast cancers, we selected eight molecular markers: EGFR, HER2, ERα, ERβ, PgR, CXCR4, p-Akt, and MVP/LRP. Representative immunohistochemical staining patterns in the presence and absence of nuclear YB-1 are shown in Fig. 2. Expression of nuclear YB-1 was detected in 30 of 73 patients (40%; nuclear YB-1 positive). Clinical and pathologic characteristics at diagnosis of the 73 patients in this study are summarized in Supplementary Table S2. There was no significant correlation between the expression of nuclear YB-1 and age (P = 0.2562), histologic grade (P = 0.1910), menopausal status (P = 0.1508), tumor size (P = 0.1478), or lymph node metastasis (P = 0.0620).

Figure 2 also shows representative examples of immunohistochemical staining for EGFR, HER2, ERα, ERβ, PgR, CXCR4, p-Akt, and MVP/LRP. There were significant correlations between the expression of nuclear YB-1 and HER2 (P = 0.0153), ERα (P = 0.0122), and CXCR4 (P = 0.0166; Table 1). By contrast, there was no significant correlation between nuclear YB-1 expression and the expression of EGFR (P = 1.0000), PgR (P = 0.0944), ERβ (P = 0.0576), p-Akt (P = 0.0521), or MVP/LRP (P = 0.0577).

Effects of nuclear YB-1 on survival and other molecular markers. The estimated product-limit survival functions of nuclear YB-1 are shown in Fig. 3*A* (overall survival) and Fig. 3*B* (progression-free survival), as well as the results of log-rank tests. Survival curves for patients with nuclear YB-1 were significantly different from those without nuclear expression (P = 0.0139 for overall survival; P = 0.0280 for progression-free survival). The results of log-rank tests for other factors are given in Table 2, showing that the tests for lymph node metastasis were significant (P = 0.0001 for overall survival; P < 0.0001 for progression-free survival).

The first eight principal components were used in the subsequent analysis, as their cumulative coefficients of variance

Table 1. Correlation between nuclear YB-1 expression and expression of eight target genes

Variables	All patients		Nuclear YB-1				*P*
			Negative		Positive		
	No. of patients	%	No. of patients	%	No. of patients	%	
EGFR							
Negative	58	79	34	79	24	80	1.0000
Positive	15	21	9	21	6	20	
HER2							
Negative	59	81	39	91	20	67	0.0153
Positive	14	19	4	9	10	33	
ERα							
Negative	24	33	9	21	15	50	0.0122
Positive	49	67	34	79	15	50	
ERβ							
Negative	18	25	7	16	11	37	0.0576
Positive	55	75	36	84	19	63	
PgR							
Negative	39	53	19	44	20	67	0.0944
Positive	34	47	24	56	10	33	
CXCR4							
Negative	29	40	12	28	17	57	0.0166
Positive	44	60	31	72	13	43	
p-Akt							
Negative	27	37	20	47	7	23	0.0521
Positive	46	63	23	53	23	77	
MVP/LRP							
Negative	41	56	20	47	21	70	0.0577
Positive	32	44	23	53	9	30	

were ~80%. Denoting the i-th principal component by PRINi, the results of Cox regression analysis were as follows. For overall survival, PRIN1 and PRIN7 were statistically significant [HR = 1.52 and $P = 0.0090$ (for PRIN1); HR = 2.06 and $P = 0.0499$ (for PRIN7); Fig. 4*A*]; and for progression-free survival, PRIN1, PRIN6, PRIN7, and PRIN8 were significant [HR = 1.59 and $P = 0.0009$ (for PRIN1); HR = 1.86 and $P = 0.0103$ (for PRIN6); HR = 2.30 and $P = 0.0078$ (for PRIN7); HR = 1.68 and $P = 0.0508$ (for PRIN8); data not shown]. PRIN1 was positively correlated with YB-1 [correlation coefficient (r) = 0.593], HER2 ($r = 0.397$), histologic grade ($r = 0.557$), tumor size ($r = 0.577$), and lymph node metastasis ($r = 0.522$). PRIN1 was negatively correlated with ERα ($r = -0.684$), PgR ($r = -0.453$), CXCR4 ($r = -0.460$), menopausal status ($r = -0.618$), and age ($r = -0.607$). This might indicate that some effect shared by YB-1, HER2, ERα, PgR, and CXCR4 leads to poor survival. PRIN7 was positively correlated with tumor size and negatively correlated with EGFR and p-Akt. Note that PRIN7 was not correlated with YB-1; this points to the existence of different mechanisms that influence survival apart from those involving PRIN1.

Stepwise variable selection was used to select the following molecular markers for graphical modeling: YB-1, HER2, ERα, ERβ, and CXCR4. Figure 4B shows the results of graphical modeling of these markers when two markers were positively correlated; a plus symbol is shown on the path, otherwise a minus symbol is shown. The relationships are indicated between markers; for example, YB-1 is related to CXCR4, ERβ, and HER2 but not directly to ERα. Note that HER2, YB-1, CXCR4, and ERα are correlated with PRIN1, emphasizing their important effects on survival.

Discussion

In this study, we assessed whether the expression of EGFR and ErbB2/HER2 was affected by YB-1 in breast cancers, as this might influence prognosis. We developed two independent approaches to identify which genes are under the control of YB-1 in human breast cancer cells. One approach involved microarray analysis, qRT-PCR, and immunoblotting to determine whether the expression of EGFRs, ERα, and other YB-1–related proteins is controlled by YB-1 in culture. The other approach consisted of immunohistochemical analysis of those protein molecules closely associated with nuclear localization of YB-1 in patients with breast cancer.

The expression of EGFR and HER2 was down-regulated by YB-1 knock-down in ERα-positive, but not ERα-negative, breast cancer cell lines, suggesting that YB-1 siRNA–induced suppression depends upon the presence of ERα. By contrast, immunohistochemical analysis showed that nuclear YB-1 expression was significantly associated with the expression of HER2 but not of EGFR. Janz et al. (6) have also reported a close association of YB-1 nuclear localization with the expression of HER2 in primary breast cancers. Moreover, Wu et al. (20) found that the introduction of a p-Akt–insensitive mutation into YB-1 markedly decreased the expressions of both EGFR and HER2, suggesting a close linkage between YB-1 and EGFR/HER2 expression in breast cancer cells in culture. YB-1 overexpression in human breast epithelial cells did not affect HER2 but caused up-regulation of EGFR, with concomitant EGF-independent phosphorylation of EGFR (18). The effect of YB-1 on EGFR and/or HER2 might depend in part on the particular cell line examined.

Oda et al. (11) found a highly significant association of p-Akt with nuclear YB-1 expression in human ovarian cancers, and both p-Akt and nuclear YB-1 expression were independent prognostic biomarkers; however, we observed no statistically significant association of p-Akt expression with nuclear YB-1 expression in our immunohistochemical analysis (Table 1). Cross-talk between growth factor receptors, such as EGFR, insulin-like growth factor (IGF) receptor, and estrogen signaling cascades occurs at the level of ERα (28, 29); this leads to activation of PI3K/Akt and ultimately to activation of ERα (30, 31). Thus, activation of ERα as well as YB-1 and its translocation to the nucleus seem to be coordinately controlled in breast cancer cells by the PI3K/Akt pathway in response to growth factors such as EGF/transforming growth factor α and IGF. PI3K/Akt activation could therefore be primarily dependent on the active state of ERα, which seems to play a major role in the nuclear translocation of activated YB-1 in ERα-positive breast cancer cells. In relation to a possible association of hormone receptors with nuclear YB-1 localization, we found that the

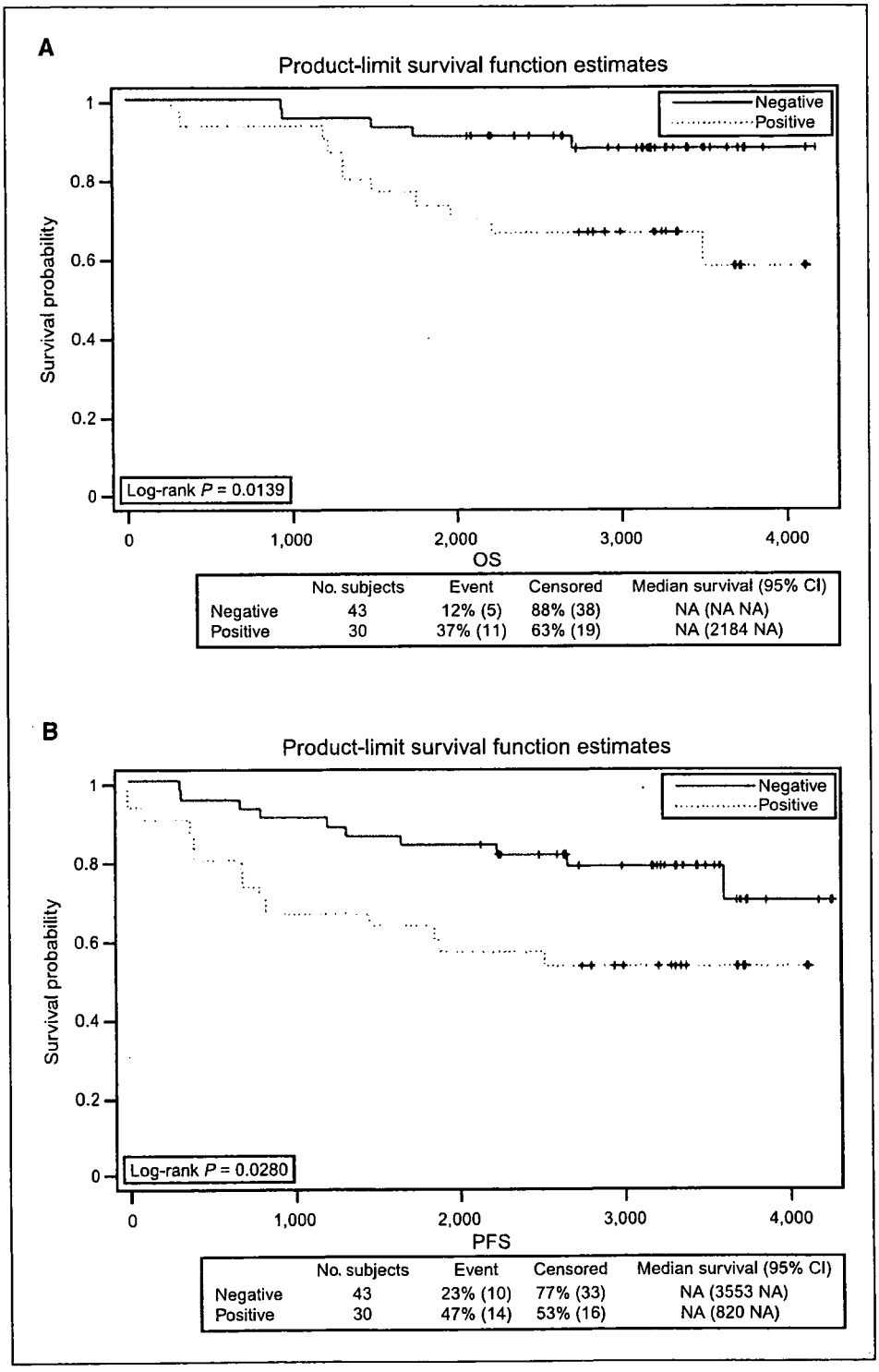

Figure 3. Kaplan-Meier overall survival (*A*) and progression-free survival (*B*) according to nuclear YB-1 expression in 73 patients with breast cancer. Nuclear expression of YB-1 has a significant predictive value for survival.

expression of ERα and ERβ was down-regulated by YB-1 knock-down. Wu and colleagues (20) have reported an inverse relationship between ERα and YB-1 in breast cancer samples. In the present study, ERα expression was inversely correlated with nuclear YB-1 localization, whereas ERβ expression was positively correlated with nuclear YB-1 localization. Like ERα, ERβ expression is closely associated with the PI3K/Akt signaling cascade (32). ERβ has emerged as an important determinant in breast cancer (33) and is a useful biomarker for breast cancer independent of ERα expression (34). The close linkage of nuclear YB-1 localization with ERβ expression points to the presence of a novel signaling pathway that could be a target for anticancer therapy in breast cancer.

We examined two targets of YB-1, MVP/LRP and CXCR4, which were identified by our expression profiling analysis. MVP/LRP expression, which is involved in drug resistance, is promoted by 5-fluorouracil and other anticancer agents in response to transcriptional activation by YB-1, suggesting a direct link between YB-1– and MVP/LRP-mediated drug resistance (35–37). MVP/LRP expression was not affected by YB-1 knock-down in ovarian cancer cells in

Table 2. Univariate analysis of patient characteristics and target gene expression regarding overall survival and progression-free survival

Variables	No. of patients	Overall survival		Progression-free survival	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Nuclear YB-1					
Negative	43	1.00		1.00	
Positive	30	3.48 (1.21–10.02)	0.0139	2.41 (1.07–5.44)	0.0280
EGFR					
Negative	58	1.00		1.00	
Positive	15	0.49 (0.11–2.17)	0.3376	0.46 (0.14–1.56)	0.2021
HER2					
Negative	59	1.00		1.00	
Positive	14	1.54 (0.50–4.77)	0.4528	2.01 (0.83–4.84)	0.1137
ERα					
Negative	24	1.00		1.00	
Positive	49	0.58 (0.21–1.54)	0.2661	0.60 (0.27–1.36)	0.2114
ER β					
Negative	18	1.00		1.00	
Positive	55	0.86 (0.27–2.66)	0.7867	1.14 (0.43–3.06)	0.7909
PgR					
Negative	39	1.00		1.00	
Positive	34	0.47 (0.16–1.36)	0.1535	1.160 (0.21–1.16)	0.0980
CXCR4					
Negative	29	1.00		1.00	
Positive	44	0.63 (0.24–1.68)	0.3509	0.59 (0.26–1.31)	0.1866
p-Akt					
Negative	27	1.00		1.00	
Positive	46	1.88 (0.61–5.83)	0.2669	1.56 (0.65–3.77)	0.3171
MVP/LRP					
Negative	41	1.00		1.00	
Positive	32	0.78 (0.28–2.15)	0.6283	0.76 (0.33–1.74)	0.5109
Age					
<56	38	1.00		1.00	
≥56	35	0.83 (0.31–2.22)	0.7032	0.63 (0.27–1.43)	0.2623
Histologic grade					
I	33	1.00		1.00	
II	20	1.56 (0.48–5.12)		1.05 (0.38–2.90)	
III	20	1.41 (0.43–4.62)	0.7364	1.42 (0.56–3.60)	0.7478
Menopausal status					
Pre	31	1.00		1.00	
Post	42	0.54 (0.20–1.45)	0.2138	0.47 (0.21–1.06)	0.0629
Tumor size					
<2 cm	30	1.00		1.00	
≥2 cm	43	2.26 (0.73–7.01)	0.1476	2.44 (0.97–6.16)	0.0508
Lymph node metastasis					
Absent	39	1.00		1.00	
Present	34	6.33 (1.80–22.29)	0.0010	8.49 (2.88–25.03)	<0.0001

Abbreviation: 95% CI, 95% confidence interval.

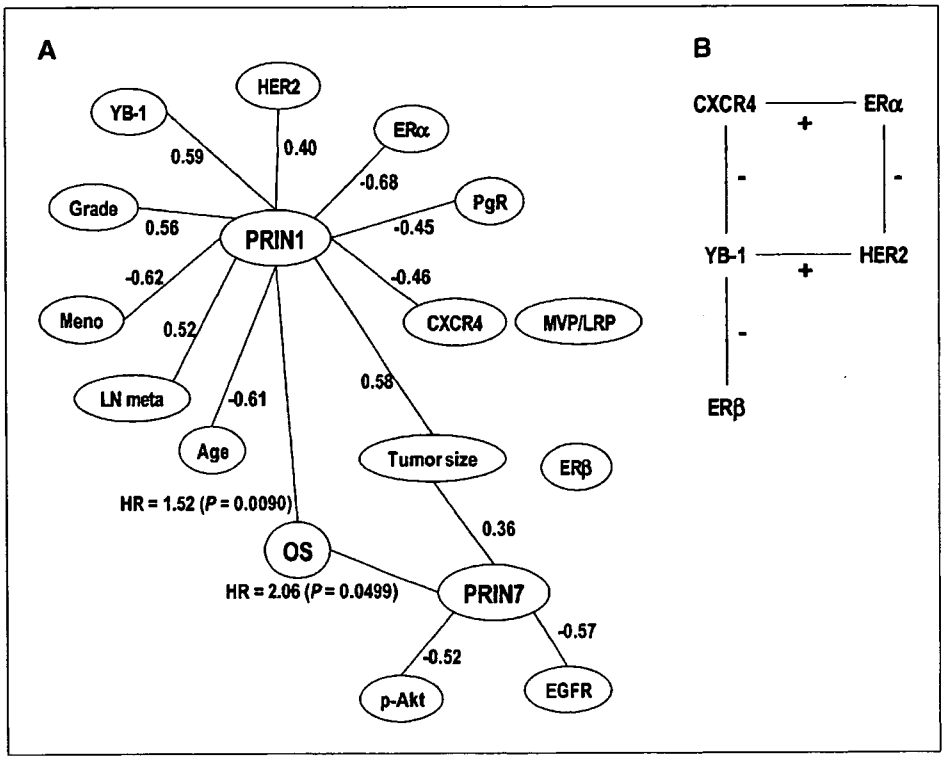

Figure 4. Statistical modeling of nuclear YB-1 localization–based network in human breast cancer. *A*, relationships among principal components, which were found significantly related to overall survival (PRIN1 and PRIN7) and clinicopathologic findings/molecular markers. Principal components and clinicopathologic findings or molecular markers are linked by a line if and only if the absolute value of correlation coefficient among them is >0.3. Each line is labeled by the correlation coefficient. *B*, relationship of molecular markers by graphical modeling incorporating with logistic regressions (+, positive correlation; −, negative correlation).

culture, although nuclear YB-1 expression and MVP/LRP expression are closely associated in patients with ovarian cancer (11, 27). CXCR4 is also known to play a critical role in the growth and metastasis of human breast cancers (38, 39). CXCR4 expression was down-regulated in YB-1 siRNA–treated ovarian cancer cells, and nuclear YB-1 expression was closely associated with CXCR4 expression in clinical samples of human ovarian cancers (11, 27). A significant positive association of nuclear YB-1 location with CXCR4 expression in breast cancer was also shown in the present study.

Nuclear localization of YB-1, in part mediated by Akt activation, thus modulates the expressions of EGFR, HER2, ERα, ERβ, and CXCR4 in breast cancer cells. YB-1–driven cell signaling of growth, survival, and hormone responses might be mainly mediated by transcriptional activation of the above-mentioned genes (1, 2); however, from our biostatistical analysis, YB-1 nuclear expression was positively associated with the expression of HER2, and negatively associated with the expressions of CXCR4 and ERβ (Fig. 4B). Moreover, ERα expression was positively correlated with CXCR4 expression and negatively correlated with HER2 expression. Although there remain inconsistencies between the data for cultured breast cancer cells and actual breast cancers with regard to the relationship between YB-1 nuclear location and the expression of other biomarkers, our biostatistical linkage map should provide important information for the development of strategies for molecular diagnosis and therapy.

In conclusion, nuclear YB-1 expression might be a prognostic marker in breast cancer. Furthermore, YB-1 plays a key role in the network annotation of genes such as HER2, CXCR4, ERα, and ERβ (Fig. 4). In addition to YB-1–mediated acquisition of multidrug resistance, the close association of nuclear YB-1 localization with HER2 expression should be considered part of the underlying mechanism. The determination of the nuclear versus cytoplasmic localization of YB-1 might provide a useful molecular indicator for personalized therapeutics of anticancer drugs targeting HER2 and/or ERα.

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