Interleukin-8 Is a Molecular Determinant of Androgen Independence and Progression in Prostate Cancer

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Abstract

The proinflammatory chemokine interleukin-8 (IL-8) is undetectable in androgen-responsive prostate cancer cells (e.g., LNCaP and LAPC-4), but it is highly expressed in androgen-independent metastatic cells, such as PC-3. In this report, we show IL-8 functions in androgen independence, chemoresistance, tumor growth, and angiogenesis. We stably transfected LNCaP and LAPC-4 cells with IL-8 cDNA and selected IL-8–secreting (IL8-S) transfectants. The IL8-S transfectants that secreted IL-8 at levels similar to that secreted by PC-3 cells (100–170 ng/10^6 cells) were characterized. Continuous or transient exposure of LNCaP and LAPC-4 cells to IL-8 reduced their dependence on androgen for growth and decreased sensitivity (>3.5×) to an antiandrogen. IL-8–induced cell proliferation was mediated through CXCR1 and was independent of androgen receptor (AR). Quantitative PCR, immunoblotting, and transfection studies showed that IL8-S cells or IL-8–treated LAPC-4 cells exhibit a 2- to 3-fold reduction in PSA and AR levels, when compared with vector transfectants. IL8-S cells expressed 2- to 3-fold higher levels of phospho-EGFR, src, Akt, and nuclear factor κB (NF-κB) and showed increased survival when treated with docetaxel. This increase was blocked by NF-κB and src inhibitors, but not by an Akt inhibitor. IL8-S transfectants displayed a 3- to 5-fold increased motility, invasion, matrix metalloproteinase-9 and vascular endothelial growth factor production. LNCaP IL8-S cells grew rapidly as tumors, with increased microvessel density and abnormal tumor vasculature when compared with the tumors derived from their vector-transfected counterparts. Therefore, IL-8 is a molecular determinant of androgen-independent prostate cancer growth and progression. [Cancer Res 2007;67(14):6854–62]

Introduction

Interleukin-8 (IL-8, CXCL-8), a member of the ELR (Glu-Leu-Arg) motif-positive (ELR+) CXC chemokine, is secreted by leukocytes and tumor cells (1). IL-8–mediated cellular response is affected by its two high-affinity cell surface receptors, CXCR1 and CXCR2, to which IL-8 cross-links and exerts biological function (2). IL-8 has diverse functions in immune surveillance, inflammation, and angiogenesis. It is known to induce motility in nonhematopoietic tumor cells, such as melanoma and breast cancer cells (3). Most metastatic and solid tumors of breast, melanoma, colon, prostate, and also several primary tumors, such as pancreatic carcinoma and glioblastoma, constitutively express IL-8 (1–6). A known function of IL-8 in tumors is enhancement of angiogenesis (4). Several reports have shown that IL-8 directly modulates endothelial cell proliferation and migration, thus promoting angiogenesis (5, 6). These studies were focused on the paracrine role of IL-8 in tumor growth, where the predominant role of IL-8 is stimulation of endothelial cell growth via increased secretion of vascular growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor. Furthermore, Sparmann et al. (7) reported that the activation of the H-Ras oncogene results in increased expression of IL-8, thus linking IL-8 to Ras-mediated tumor promotion and metastasis.

It has been shown that androgen and estrogen suppress the transcription of IL-8, and the production increases upon androgen withdrawal (8, 9). In addition, circulating levels of IL-8 are increased in advanced prostate cancer at a stage when the tumors no longer respond to antiandrogens (10, 11). In androgen-independent prostate cancer cells, IL-8 expression enhances tumorigenicity and metastasis (12, 13). However, it is unknown whether IL-8 is a cause or consequence of androgen independence and metastatic tumor progression. Furthermore, the mechanism by which IL-8 contributes to prostate cancer cell growth and metastasis has not been established.

We hypothesized that endogenous IL-8 production, either constitutive or induced by inflammatory agents, triggers down-regulation of androgen-mediated proliferative pathway, which, in turn, helps prostate cancer cells to overcome androgen-depletion therapy. Furthermore, changing the phenotype of androgen-responsive cells from IL-8 nonproducing to constitutively expressing of IL-8, causes activation of survival factors, leading to increased survival following androgen ablation or chemotherapy.

We report here a systematic investigation of the consequences of continuous IL-8 expression in two androgen-responsive cell lines, which do not express IL-8 under normal conditions (9, 12). The results of our investigation regarding the androgen sensitivity, proliferation, and chemoresistance are presented.

Materials and Methods

Cell lines. Androgen-responsive human prostate cancer cell line LAPC-4 was provided by Dr. Charles L Sawyers (University of California at Los Angeles, Los Angeles, CA) and was maintained in Iscove's medium (Invitrogen Inc.) with 7.5% fetal bovine serum (FBS) and 1 nmol/L dihydrotestosterone. LNCaP and PC-3 cells were maintained in RPMI + 10% FBS (14).

Reagents. Recombinant human CXCL8/IL-8, ELISA kits and antibodies for CXCR-1 and CXCR-2 were from R&D Systems. Docetaxel (Taxotere, TXTR) was a gift from Sanofi-Aventis Pharmaceuticals. Parthenolide (Pa),

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

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PP2, and LY294002 were purchased from EMD Bioscience. Bicalutamide (Casodex, AstraZeneca-USA) was from University of Miami Pharmacy.

Stable cell line generation. IL-8 cDNA containing the entire coding region was amplified by reverse transcription-PCR (RT-PCR) using total RNA from PC3 cells and the following PCR primers: IL8-L, 5′-AGGAACCATCTCACTGTGTG-3′; IL8-R, 5′-GGCATCTTCACTGATTCTTG-3′. The 346-bp cDNA was cloned into a bidirectional eukaryotic expression vector pEF6/V5-His TOPO (Invitrogen). Empty plasmid vector or the plasmid containing IL-8 sense (IL8-S) cDNA was transfected into LNCaP and LAPC-4 cells using Effectine reagent (Qiagen; ref. 15). Stable transfectants were selected in growth medium containing 5 μg/mL Blasticidin.

ELISA. Conditioned media of the 72-h cell cultures were assayed for IL-8, prostate-specific antigen (PSA) and VEGF by ELISA (Research Diagnostics Inc.; R&D Systems), and their levels were normalized either to total protein or cell number. Data are presented as mean ± SE from three separate experiments.

Real-time PCR. Total RNA from vector, IL8-S clones, and PC3 cells was isolated and subjected to reverse transcription and quantitative-real-time PCR using the Bio-Rad iCycler iQ real-time PCR system (Bio-Rad) and following primers: IL-8, IL8-L: 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′; IL8-R, 5′-TCTCAGCCCTCTTCAAAAACTTCT-3′; CXCR 1-L, 5′-AGCCAGATCACCTTCCACACACAA-3′; CXCR1-R, 5′-GCAAGGAGTTCTTGGCACGTCATT-3′; CXCR2-L, 5′-AGCAGGAAGATGAGGACAACAGCA-3′; CXCR2-R: 5′-ACAATACAGCAAACTGGCGGATGC-3′; AR-L; 5′-CCTGGCTTCCGCAACTTACAC-3′; AR-R, 5′-GGACTTGTGCATGCGGTACTCA-3′. As a control, each cDNA sample was simultaneously subjected to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or actin PCR using the primers: GAPDH-L: 5′-CCACCCATGGCAAATTCCATGGCA-3′; GAPDH-R: 5′-TCTAGACGGCAGGTCA GGTCCACC-3′; β-actin–L: 5′-CAACTGGGACGACATGGA-3′; β-actin-R: 5′-GTTGGCCTTGGGGTTCAG-3′; The threshold cycle (Ct) of each sample was determined, and the relative level of a transcript ($2^{\Delta Ct}$) was calculated by obtaining ΔCt (test Ct − GAPDH Ct) and then expressed as arbitrary units ($1/2^{\Delta Ct} \times 100$) = fold difference (FD; refs. 16).

[^{3}H]-thymidine incorporation (DNA synthesis) assay. Cultures of 24 h of vector or IL8-S transfectants incubated in 10% charcoal-dextran–treated FBS (CDFBS; Hyclone Inc.) with or without 1 nmol/L dihydrotestosterone were treated with various test agents, and DNA synthesis activity was assayed by 2 h pulse labeling with [^{3}H]-thymidine (0.1 μCi/mL, ICN International Inc.) as described before (18).

Cell viability assay. Twenty-four-hour cultures of vector and IL8-S clones were treated with various doses of TXTR for 72 h. During the first 24 h of incubation, DMSO (diluent) or parthenolide (1 μmol/L), PP2 (5 μmol/L), or LY294002 (5 μmol/L) was added to the cultures, and cell viability was quantified by colorimetric thiozolyl blue [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] reduction assay (14).

Immunoblot analysis and zymography. Whole cell extracts were subjected to immunoblot analysis using antibodies p-Src (Tyr^{416}), Src, p-NF-κB p65 (Ser^{536}), NF-κB p65, p-Akt (Ser^{473}), Akt, cleaved poly(ADP-ribose) polymerase (PARP; Asp^{214}), p-EGFR (Tyr^{1173}; Cell Signaling Inc.) and AR Lab Vision Corporation and actin (BD PharMingen). Protein blots were visualized on X-ray films using chemiluminescence reagents (ECL Plus, GE/Amersham).

Matrix metalloproteinase (MMP) levels in 72-h serum-free culture conditioned media of vector and IL8-S transfectants were analyzed by zymography or immunoblotting as described previously (14).

Luciferase reporter assays. pNF-κB-luc (p-NF-κB-Luc cis reporter plasmid) was from Stratagene. This reporter gene construct contains five repeats of a consensus NF-κB binding site. Cells were cotransfected with pNF-κB-luc and pRL-TK *Renilla* using Effectene kit (Qiagen). The reporter activity was assayed 48 h after transfection using the Promega Dual-Glo assay kit (Promega Scientific). Relative Light Units (activity, RLU) were normalized to TK-*Renilla* luminescence.

Chemoinvasion and chemotactic motility assays. We used dual-chamber transwell plates (Corning Labware Inc.) containing porous top chambers [12-μm pore with Matrigel coating for chemo-invasion assay (invasion assay) or 8-μm pore without Matrigel coating for chemotactic motility assay (motility assay)]. Cells were plated in the top chamber ($4 \times 10^5/cm^2$) in serum-free medium containing the insulin, transferrin, and selenium (ITS) premix (Sigma Chemical Co.). For the motility assay, the bottom chamber contained human lung fibroblast conditioned medium (14), whereas for the invasion assay, the bottom chamber had medium plus 10% FBS. Following incubation (invasion assay: 48 h; motility assay: 18 h), the invasive or motility activity was expressed as the % of cells in the bottom chamber, determined by MTT assay, as described before (14).

Tumor growth studies. Vector and IL8-S transfectants (5×10^6 cells in 50% Matrigel) were implanted s.c. on the dorsal flank of 6–8-week-old athymic male mice (eight mice per group, Charles River Laboratories). Tumor growth was monitored over time by measuring tumor volumes with a caliper, and the volume was approximated to an ellipsoid (14). At necropsy, blood was collected to measure PSA and IL-8 levels. The tumors were fixed in formalin, and histology was done at Charles River Laboratories.

Microvessel density determination. Microvessel density (MVD) in tumor specimens were determined using a rat anti-mouse CD34 immunoglobulin (3.1 μg/mL; BD Bioscience) and quantitated as described before (15, 17).

Results

Prostate cancer cells constitutively express IL-8 receptors regardless of their IL-8 expression. Because there is significant ambivalence about the expression pattern of IL-8 and its receptors in prostate cancer (19–25), we investigated the expression of IL-8 and its receptors (i.e., CXCR1 and CXCR2) by real-time RT-PCR. We found little IL-8 mRNA expression in LNCaP and LAPC-4 compared with PC-3 cells [FD = 0.041 ± 0.02 (LNCaP), 0.32 ± 0.04 (LAPC-4) versus 19 ± 4.0 (PC-3)]. However, all three prostate cell lines express mRNAs for both IL-8 receptors at comparable levels (CXCR1: 0.6–1.3; CXCR2: 0.4–0.83; see Supplementary Table S1).

Because both androgen-responsive cells, LNCaP and LAPC-4, do not express IL-8, we stably transfected these cells with a full-length (IL8-S) cDNA construct or the vector-only construct (vector or V). We initially selected 15 LNCaP and 9 LAPC-4 IL-8 secreting stable clones, and the data on three clones of each cell line are shown here. Figure 1*A* shows that IL8-S clones secrete 150- to 200-fold more IL-8 than the respective vector transfectants of LAPC-4 and LNCaP cells, and the secreted IL-8 levels are comparable with that of PC3 cells. Furthermore, real-time RT-PCR confirmed that the IL-8 mRNA expression in IL8-S clones was comparable with that by PC3 cells (data not shown).

IL-8 stimulates proliferation in androgen-responsive cells. Lee et al. (25) reported previously that IL-8 causes proliferation of androgen-deprived LNCaP cells, but no data on LAPC-4, an androgen-responsive cell line expressing wild-type AR (26). As shown in Fig. 1*B-i*, we observed a dose-dependent proliferation of IL-8–stimulated proliferation of both LNCaP and LAPC-4 cells in the absence of DHT. The IL8-induced growth of LNCaP and LAPC4 cells was statistically significant (Dunn's test; $P < 0.001$). Therefore, exogenous addition of IL-8 promotes the growth of androgen-responsive cells in the absence of androgen.

We next examined whether the constitutive expression of IL-8 accelerates proliferation (DNA synthesis activity) of LAPC-4 cells. The DNA synthesis activity of LACP-4 cells in the absence of DHT is 6.7-fold lower when compared with that in the presence of DHT ($P < 0.001$; unpaired *t* test; Fig. 1*B-ii*). In contrast, the proliferation rate of IL8-S clones in the absence of DHT is 4- to 5-fold higher than that of vector transfectants (Fig. 1*B-ii*; $P < 0.01$, Tukey's test). The growth rate of IL8-S clones increased about 2-fold in the presence of DHT and was comparable with that of vector transfectants grown in the presence of DHT. This study was further confirmed by cell counting following a 7-day treatment of

vector and IL8-S transfectants with IL-8 in the presence or absence of DHT. Similar results were obtained by cell counting (data not shown). Therefore, constitutive expression of IL-8 promotes androgen-independent cell proliferation.

CXCR1-mediated IL-8 induced cell proliferation. Because CXCR1 and CXCR2 contribute to cell proliferation in endothelial cells (27), we investigated for such properties in IL8-expressing prostate cancer cells. As shown in Fig. 1*C-i*, blocking IL-8 binding to CXCR1 by anti-CXCR1 antibody caused inhibition of cell proliferation in LAPC-4 IL8-S and PC3 cells. However, blocking IL-8 binding to CXCR1 does not alter DNA synthesis in vector transfectants, although these cells express CXCR1. Inhibition of IL-8 binding to CXCR2 by anti-CXCR2 antibody did not alter the proliferation of any of the cell lines (Fig. 1*C-ii*) suggesting that IL-8–induced growth is mediated by CXCR1.

Because constitutive expression of IL-8 in tumor-associated brain endothelial cells stimulates VEGF expression (28), we measured VEGF levels (VEFG-A) in the culture-conditioned media of vector and IL8-S transfectants. As shown in Fig. 1*D*, VEGF-A secretion is increased 2- to 4-fold in IL8-S transfectants when compared with vector transfectants of LAPC-4 and LNCaP cells ($P < 0.001$; Tukey's test). Furthermore, the VEGF levels in IL8-S transfectants of both cell lines are comparable with that of PC-3.

IL-8 expression reduces sensitivity to antiandrogen, bicalutamide. Because both exogenous and endogenous IL-8 stimulated proliferation of LAPC-4 cells under androgen ablation, we hypothesized that IL-8 may alter the sensitivity of LNCaP and LAPC-4 cells to antiandrogens, such as bicalutamide. LNCaP cells are not as sensitive to bicalutamide as other androgen-responsive

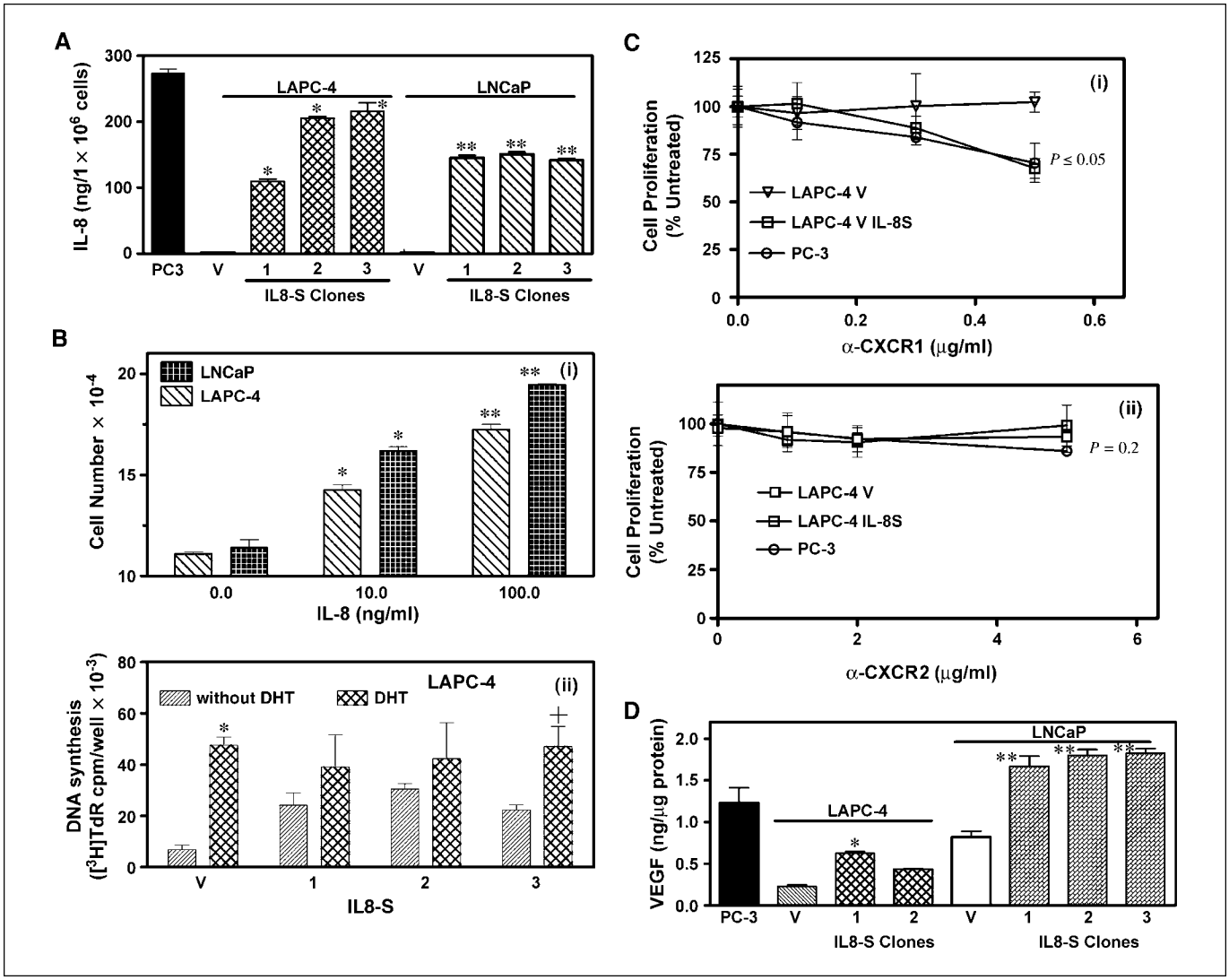

Figure 1. Characterization of IL8-S and vector transfectants. *A,* IL-8 production from IL8-S and vector clones of LAPC-4 and LNCaP was measured by ELISA, and the results were normalized to cell numbers. *B, i,* LAPC-4 and LNCaP cells were treated with various concentrations of IL-8 CDFBS medium without DHT for 72 h, and the growth rate was calculated by counting total number of cells/time point. *ii,* LAPC-4 vector and IL8-S clones were cultured in CDFBS medium with or without 1 nmol/L DHT for 3 d, and DNA synthesis was measured by [^{3}H]-thymidine incorporation assay. *C,* LAPC-4 vector, IL8-S clones, and PC3 cells (positive control) were treated with either anti-CXCR1 (*i*) or anti-CXCR2 (*ii*) neutralizing antibody for 3 d, and DNA synthesis inhibition was measured by [^{3}H]-thymidine incorporation assay (14). *D,* LAPC-4 and LNCaP transfectants were cultured for 72 h, and the cultured media were assayed for VEGF by an ELISA. VEGF levels were normalized to total protein concentration. *A–D, columns* and *points,* representative means of two or three independent experiments done in triplicate; *bars,* SE. * or **, significant differences ($P < 0.05$) in between vector and IL8-S transfectants of respective cell lines as determined by pairwise Student's *t* test.

cells (e.g., LAPC-4), due to a mutation in the ligand-binding domain of AR (26, 29). As shown in Fig. 2*A*, bicalutamide inhibited DNA synthesis in LAPC-4 and LNCaP vector transfectants, but not that of IL8-S transfectants, suggesting that IL8-S clones are less dependent on mitogenic signaling by AR.

To determine why IL8-S clones were less dependent on androgen, we examined whether IL8-S cells are either delinked from AR signaling and/or AR levels are down-regulated in these cells. As a measure of AR activity, we measured PSA levels. As shown in Fig. 2*B*, PSA protein expression is undetectable in both transfectants when cultured without DHT. However, PSA expression (221 ± 23.6 ng/10^5 cells/mL) is strongly induced in the presence of DHT due to AR signaling (Fig. 2*B*). In IL8-S cells treated with DHT, PSA levels were 70% lower (81.3 ± 6.8 ng/10^5 cells/mL) than that of vector transfectants (Fig. 2*B*). Similar results were also obtained in LNCaP IL8-S transfectants. These results show that constitutive expression of IL-8 inhibits AR signaling, and this may result in reduced sensitivity to antiandrogens and less secretion of PSA with androgen stimulation.

Constitutive expression of IL-8 down-regulates AR. We next examined AR protein levels in vector and IL8-S transfectants. As shown in Fig. 2*C-a*, AR levels in IL8-S cells are 2-fold lower than the levels in vector transfectant. AR protein level is ~3-fold higher in LAPC-4 vector transfectant in the presence of DHT for 4 h when compared with AR levels in the absence of DHT. In contrast, in IL8-S cells, there is no increase in AR levels when the cells are grown in the presence of DHT (Fig. 2*C-a*). Whether AR was being degraded via ubiquitin-mediated 26S proteasome pathway in IL8-S cells was examined in cell lysates prepared from vector and IL8-S cells treated with MG132 in the presence of DHT. Consistent with AR activation followed by its nuclear localization and stabilization, there is no further increase in AR levels in DHT-treated vector cells when treated with MG132. However, MG132 did not increase AR levels in DHT-treated or untreated IL8-S cells, suggesting that AR degradation is unlikely a cause of observed lower AR levels in IL8-S cells. To determine whether exogenous IL-8 also down-regulates AR, we assayed AR levels in LAPC-4 treated with IL-8 by immunoblotting. As shown in Fig. 2*C-b*, IL-8 treatment decreases AR levels at 4 h and remain low even at 8 h. This IL8-induced decrease in AR levels could not be reversed by coincubation of LAPC-4 cells with IL-8 and MG132 (data not shown). We next determined whether the decrease in AR levels in IL8-S transfectant is due to a decrease in AR transcription. As shown in Fig. 2*D*, AR mRNA levels in vector transfectant are 2-fold higher than AR mRNA levels in IL8-S cells. Furthermore, in the presence of DHT, there is an increase in AR transcript level in vector cells. However, AR transcript levels do not appreciably change in IL8-S cells following DHT treatment ($P < 0.05$). These results show that

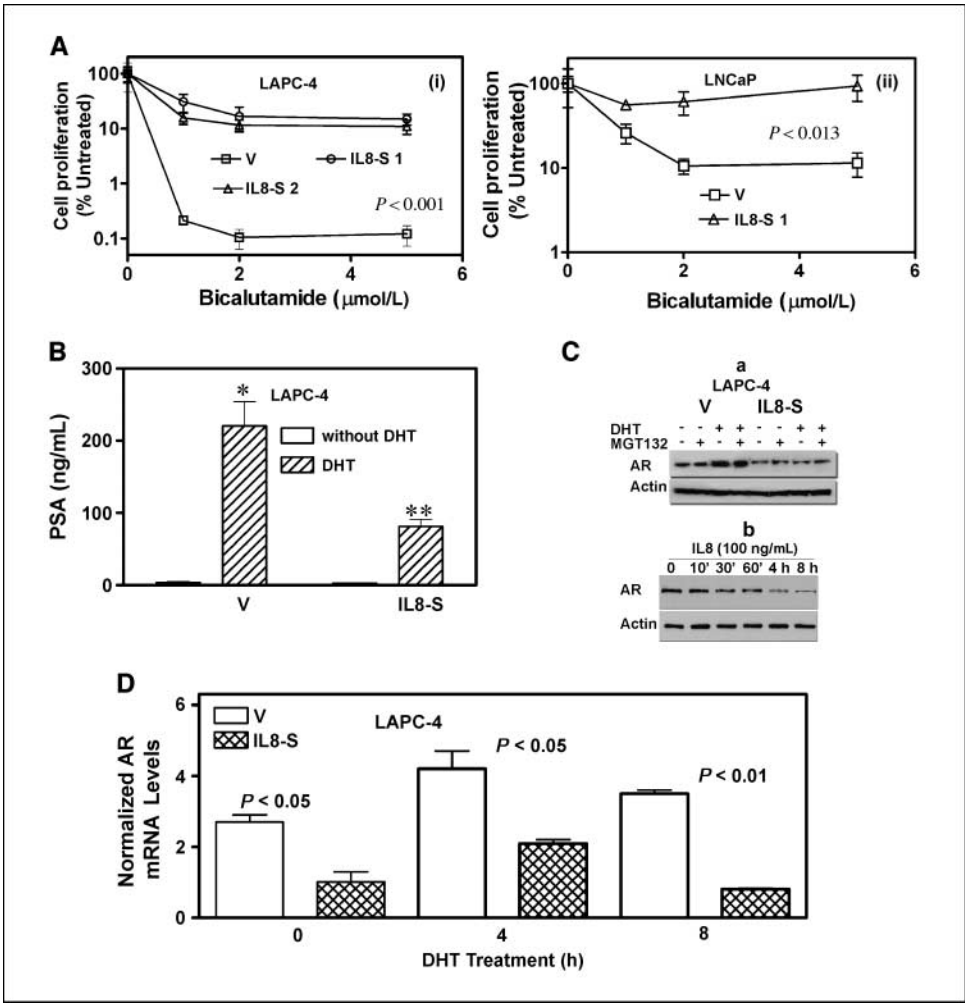

Figure 2. Effect of IL-8 on androgen-independent growth, AR levels, and signaling. *A,* LAPC-4 and LNCaP transfectants were treated with various concentrations of bicalutamide for 3 d, and DNA synthesis was measured by [^{3}H]-thymidine incorporation assay. *Points,* mean; *bars,* SE. *B,* PSA measurement in culture-conditioned media of LAPC-4 vector and IL8-S clones cultured in CDFBS media with or without DHT for 48 h. In (*A*) and (*B*), the data are from two or three individual experiments in triplicates. *C, a,* immunoblot analysis of AR in LAPC-4 transfectants cultured in media with or without DHT for 48 h, either in the presence or absence of MG132. *b,* LAPC-4 cells were treated with 100 ng/mL IL-8 for various time points, and AR protein levels were examined by immunoblotting. Loading control, actin. *D,* real-time PCR analysis of AR in vector and IL8-S transfectants. LAPC-4 vector and IL8-S cells were treated with DHT for 0, 4, and 8 h and then subjected to real-time RT-PCR. Normalized transcript levels were calculated as $1/2^{\Delta Ct} \times 100$. Each time point for each cell line represents duplicate determination. In (*B*), * or **, $P < 0.05$, significant difference by *t* test.

IL-8–induced increase in the proliferation is very likely independent of AR-mediated signaling.

IL-8 causes up-regulation of survival factors in prostate cells. IL-8 has been shown to activate extracellular signal-regulated kinase 1 and 2 (ERK 1/2), suggesting that IL-8 activates the phosphoinositide-3-kinase (PI3K) and mitogen-activated protein (MAP) kinase (MAPK) cascade (30, 31). In LNCaP cells, it has been reported that exogenous IL-8 up-regulates src and ERK activity (25). Thus, we hypothesized that autocrine expression of IL-8 in androgen-responsive cells may not only stimulate MAPK activity, but also up-regulate other survival factors, such as Akt and nuclear factor κB (NF-κB). As shown in Fig. 3*A*, p-EGFR, p-src, p-Akt, and NFκB p-p65 levels are increased in IL8-S clones of both LNCaP and LAPC-4, when compared with the respective vector transfectants. The changes in p-p65 and p-Akt levels are relatively less in LNCaP when compared with those in LAPC-4 cells. Because there is a close link between IL-8 and activation of NFκB, we investigated whether exogenous IL-8 treatment also increases p-p65 (NFκB) levels. As shown in Fig. 3*B*, IL-8 treatment increases NFκB p-p65 levels; the increase is detectable as early as within 1 h of IL-8 treatment and reaches a maximum after 4 h of treatment (Fig. 3*B*). We next used a luciferase reporter assay to measure the transcriptional activity of NFκB in IL8-S and vector transfectants of LAPC-4 cells. After 48 h transfection with a NFκB-reporter construct, we observed a 5.7-fold increase of pNFκB-luciferase activity in LAPC-4 IL8-S cells when compared with that in the vector transfectant (Fig. 3*C*). Similar results were also observed in NFκB reporter transfectants of LNCaP IL8-S cells (data not shown).

IL-8 increases resistance to cytotoxic drugs by up-regulation of survival factors. As shown in Fig. 4*A*, IL8-S clones of LAPC-4 are significantly more resistant to TXTR compared with vector transfectants cells (IC_{50} for vector: 2.95 ± ng/mL; IC_{50} for IL8-S: 7.9 ± 0.4 ng/mL). Next, we investigated whether systematic disruption of survival factor up-regulation decreases chemoresistance. LAPC-4 vector and IL8-S clones were treated with TXTR in combination with inhibitors of the survival factors (PP2: Src inhibitor; LY294002: PI3K inhibitor; parthenolide: NFκB inhibitor).

As shown in Fig. 4*A-i* and *ii*, blockage of NFκB and src by their respective inhibitors resulted in a significant decrease in the IC_{50} of TXTR in LAPC-4 IL8-S cells. However, inhibition of Akt activity by LY 294002 did not affect the survival in both vector and IL8-S cells after TXTR treatment (Fig. 4*A-iii*), suggesting that activation of Akt in LAPC-4 may not contribute to chemoresistance. The differences in the sensitivity of vector and IL8-S clones to TXTR were also confirmed by measuring cleaved PARP (Fig. 4*B-a* and *b*). However, inhibition of NFκB or src activity by respective inhibitors resulted in increased cleaved PARP levels in IL8-S cells following TXTR treatment to levels similar to vector cells. These results show that activation of src and NFκB may be a mechanism of IL-8–induced chemoresistance.

IL-8 enhances invasive characteristics. As shown in Fig. 5*A*, IL8-S transfectants showed 4- to 5-fold more chemotactic motility when compared with vector transfectants. The chemotactic motility of LAPC-4 IL8-S transfectants is comparable with that of PC3 cells, which secrete IL-8. Similar analysis of LNCaP cells also showed that LNCaP IL8-S cells are ~2.5 times more motile than LNCaP vector cells (data not shown). Next, we examined the invasive potential of various transfectants by Matrigel invasion assay. As shown in Fig. 5*B*, invasive activity of IL8-S transfectants

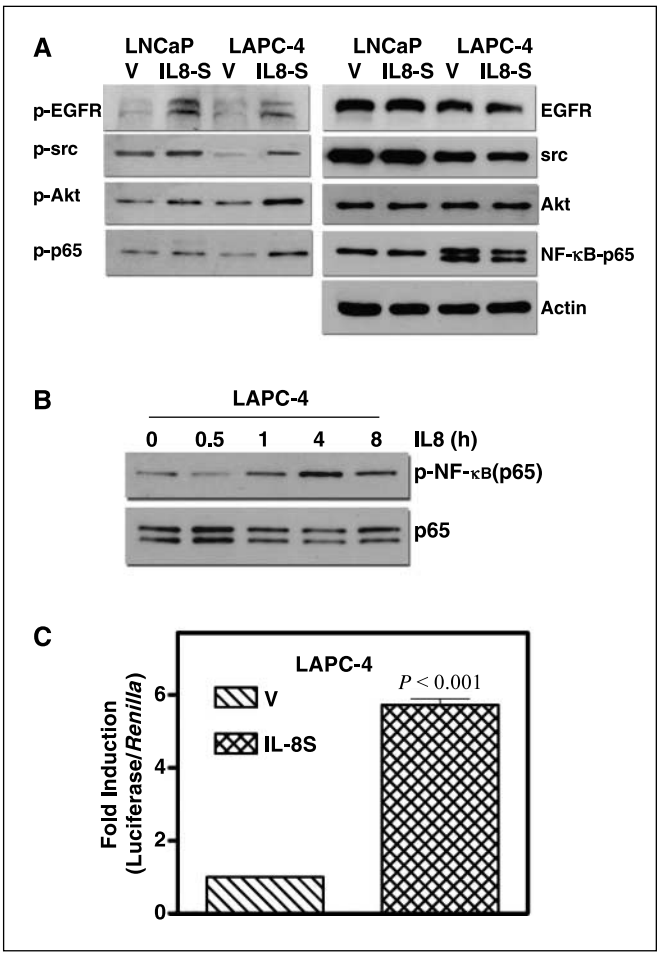

Figure 3. Analysis of survival factors in LNCaP and LAPC-4 transfectants. *A,* cell lysates of vector and IL8-S transfectants were analyzed by immunoblotting using antiphospho- or anti-total EGFR, Src, Akt, and NF-κB (p-65) antibodies. *B,* LAPC-4 cells treated for various periods of time with IL-8 (100 ng/mL) in 1% FBS-containing medium were analyzed by immunoblotting for phospho-NF-κB (p-65) levels. In (*A*) and (*B*), actin level was used as a loading control. *C,* LAPC-4 transfectants were transiently transfected with 5× κB-luc (Stratagene) and cultured in CDFBS media without DHT. After 48 h, promoter activity was assayed by luciferase assay and normalized to *Renilla* activity (Promega).

was similar to that of PC3 cells, and it is 2- to 2.5-fold higher than that of vector transfectant.

Examination of invasive-promoting proteinases, such as gelatinases, showed that MMP-9 is not detected in vector cells, but is elevated in IL8-S cells and also in PC3 (Fig. 5*C-a*). Furthermore, both pro- and active MMP-9 are detected in IL8-S and PC3 cells. However, unlike MMP-9, MMP-2 secretion is not increased appreciably in IL-8 and PC3 cells, when compared with the vector transfectant. Increase in MMP-9 levels in LAPC-4 IL8-S compared with vector cells was further confirmed by immunoblotting (Fig. 5*C-b*).

Enhanced tumor growth and increased angiogenic activity in IL8-S LNCaP xenografts. Our observation of IL8-S cells *in vitro* suggested an increase in their tumor growth and metastatic potential *in vivo*. To test this possibility, IL8-S and vector transfectants of LNCaP cells were injected with Matrigel into athymic male mice. Tumor incidence (percentage of animals developing palpable tumors), latency (time to develop palpable tumor), and growth rate were monitored for 60 days.

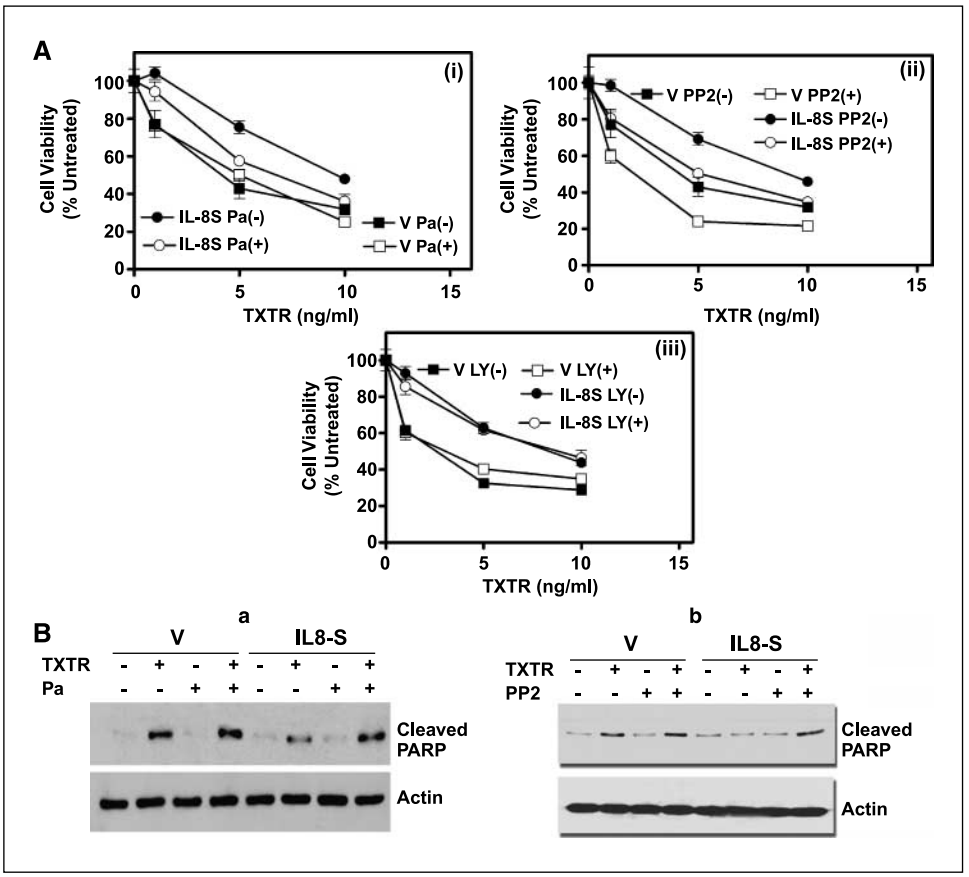

Figure 4. Analysis of IL-8–induced drug resistance in LAPC-4 transfectants. *A,* vector and IL8-S transfectants were cotreated with various doses of TXTR for 72 h and inhibitors for the first 24 h. Inhibitors used are (*i*) NFκB inhibitor parthenolide (*Pa*, 1 μmol/L), (*ii*) Src inhibitor PP2 (5 μmol/L), and (*iii*) PI3K inhibitor LY294002 (*LY*, 5 μmol/L). Cell viability after treatment was monitored by MTT assay. *Points,* mean of three individual experiments in triplicates; *bars,* SE. *B,* cleaved PARP levels in the cell lysates of transfectants were treated with TXTR ± Pa (*a*) or PP2 (*b*) were analyzed by immunoblotting. Loading control, actin.

There was no significant difference with respect to tumor incidence between LNCaP vector (66%) and IL8-S (80%) cells. However, IL8-S tumors grew about 30% to 35% faster (Fig. 6*A* and *B*). In addition, in the IL8-S group, 62.5% (5/8) of the animals developed highly hemorrhagic tumors (Fig. 6*A*). Tumor histology showed the presence of large vessel collapse and disorganized vessel formation in IL8-S tumors, whereas no necrosis was observed in vector tumors.

We analyzed MVD in tumors that were not overtly necrotic or hemorrhagic by immunohistochemical staining using mouse CD34 antibody. As shown in Fig. 6*C*, there was a significant increase in MVD and in the length of microvessels in IL8-S tumors, when compared with vector tumors. MVD in IL8-S tumors was 2.5-fold higher than in vector tumors (Fig. 6*D*). Furthermore, the length of microvessels in IL8-S tumor was 3.4-fold higher than in vector tumors ($P < 0.05$). These results show that IL-8 expression increases tumor growth and angiogenesis.

Measurement of circulating IL-8 levels in serum, collected at necropsy, showed significant amount of IL-8 (7 ± 2.2 ng/mL) in mouse serum in LNCaP-IL8-S tumor-bearing mice. IL-8 was not detected in normal mouse sera and sera from LNCaP-vector tumor-bearing animals, as mice are not known to produce IL-8 or its homologues (32).

Discussion

Several studies show that IL-8 modulates proliferation and migration of tumor cells, including prostate cancer cells (2, 6, 7, 12, 13). However, these studies were focused on the role of IL-8 on androgen-dependent cells under androgen withdrawal condition or in androgen-independent cells (13, 25). To our knowledge, no report has appeared on the cross-talk between AR and IL-8 signaling during androgen stimulation. Thus, we expected that IL-8 expression will stimulate cell proliferation regardless of androgen availability. We observed that either the exogenous addition of IL-8, or constitutive expression of IL-8, stimulated the growth of androgen-responsive cells only under androgen-depletion condition. This suggests that perhaps IL-8 may help prostate cancer cells to become androgen independent upon androgen withdrawal. This is further illustrated by the observed insensitivity of IL8-S transfectants to AR antagonists such as bicalutamide (Fig. 2*A*). Autocrine or paracrine production of IL-8 seems to exhibit a dominant effect rather than the IL-8 receptors (CXCR1 and CXCR2, see Supplementary Table S1) in modulating cellular functions because both IL-8S and vectors exhibited the same level of expression of both CXCR1 and CXCR2 mRNA as that of PC-3. Furthermore, growth inhibition by the anti-CXCR1 antibody in PC-3 cells but not in LNCaP-v cells also suggests that IL-8 is a mediator of cell proliferation.

The IL-8–induced growth of androgen-responsive cells, in the absence of androgen, seems to be independent of AR. This is because AR protein levels are lower in IL8-S cells when compared with vector cells. Furthermore, unlike the vector transfectants, where AR levels increase when the cells are grown in the presence of DHT, AR levels do not increase in IL8-S transfectants grown in the presence of DHT. Moreover, PSA production in IL8-S cells is

70% lower than in vector transfectant, when cells are grown in the presence of DHT, suggesting that AR-mediated signaling is attenuated in the presence of IL-8. The decreased AR levels in cells treated with IL-8 in the presence of DHT are in part due to a decrease in AR mRNA levels because AR transcript levels are 2- to 4-fold lower in IL-8 cells when compared with vector cells (Fig. 2*D*). Although IL-8 seems to regulate AR levels, at present, it is not clear whether AR-independent growth results in the up-regulation of IL-8 levels. It has been shown, however, that in C4-2 cells (an androgen-independent subline of LNCaP), IL-8 transcript levels are lower than the levels in LNCaP; however, the mRNA levels do not correlate with protein levels (19).

Lee et al. (25) showed that short-term exposure of LNCaP cells to IL-8 (30 and 120 min treatment) caused AR binding to the androgen response element (ARE) present in the PSA promoter, via src activation, and they concluded that exogenous IL-8 activates AR signaling. However, our findings show that longer exposure of androgen-responsive cells to IL-8 (4 and 8 h) causes AR down-regulation and inhibits AR-induced PSA promoter activity (Fig. 2*C-b*). These findings suggest that whereas shorter IL-8 treatment may cause activation of AR via src, continuous exposure down-regulates AR protein levels by inhibition of AR transcription. Consistent with this hypothesis, our results show that IL-8 activates the src pathway by inducing src phosphorylation.

A significant finding of our studies is that endogenous IL-8 causes constitutive activation of NFκB (p65). It has been reported that NFκB stimulates IL-8 production (1), whereas there is limited understanding on how IL-8 causes activation of NFκB. A well-established mechanism of NFκB activation involves the activation of phosphorylated Akt (33, 34). In this study, we found that endogenous IL-8 caused phosphorylation of epithelial growth factor receptor (EGFR), src, Akt, and NFκB. However, inhibition of Akt phosphorylation did not prevent IL-8–mediated NFκB activity; this is because whereas parthenolide reversed the increased survival of IL8-S cells in the presence of TXTR, LY22909 did not reverse this. Venkatakrishnan et al. (35) have reported the involvement of EGFR in the signaling of CXCR-1/2. Therefore, it is possible that IL-8 expression causes activation of both MAPK cascade and Akt by activating protein kinase C and EGFR, leading to the activation of NFκB in androgen-responsive cells.

It is significant to note that the activation of src and NFκB by IL-8 contribute to the survival of IL-8–expressing cells against the chemotherapeutic drug TXTR (Fig. 4). These observations might open a new opportunity to enhance the therapeutic efficacy of chemotherapeutic drugs in resistant tumor cells by the combination of IL-8 antagonists or antagonists of activators of NFκB. This may be particularly significant in the treatment of androgen-independent prostate cancer where response to standard chemotherapy using TXTR leads to significant but only a short-term therapeutic response (36).

Binding of IL-8 to CXCR2 has been shown to promote angiogenesis (27). We found that LNCaP cells expressing IL-8 when injected s.c. into athymic mice produce rapidly growing hemorrhagic tumors with wide ranging angiogenic activity from high MVD to vascular rupture, leading to necrosis (Fig. 6). Because no known homologue of IL-8 exists in mice, the murine endothelial cells respond to human vascular endothelial growth factor (hVEGF; ref. 37), which in this case is produced by LNCaP IL8-S cells. Rapid growth of LNCaP IL8-S cells, with increased production of IL-8, plausibly leads to abnormal proliferation of murine endothelial cells, which, in turn, may cause abnormally leaky tumor vasculature.

In conclusion, the present study provides evidence that (*a*) IL-8 contributes to cell proliferation under androgen depletion, but not under androgen stimulation; (*b*) IL-8 down-regulates AR in LAPC-4; this is the first evidence to show such a property; (*c*)

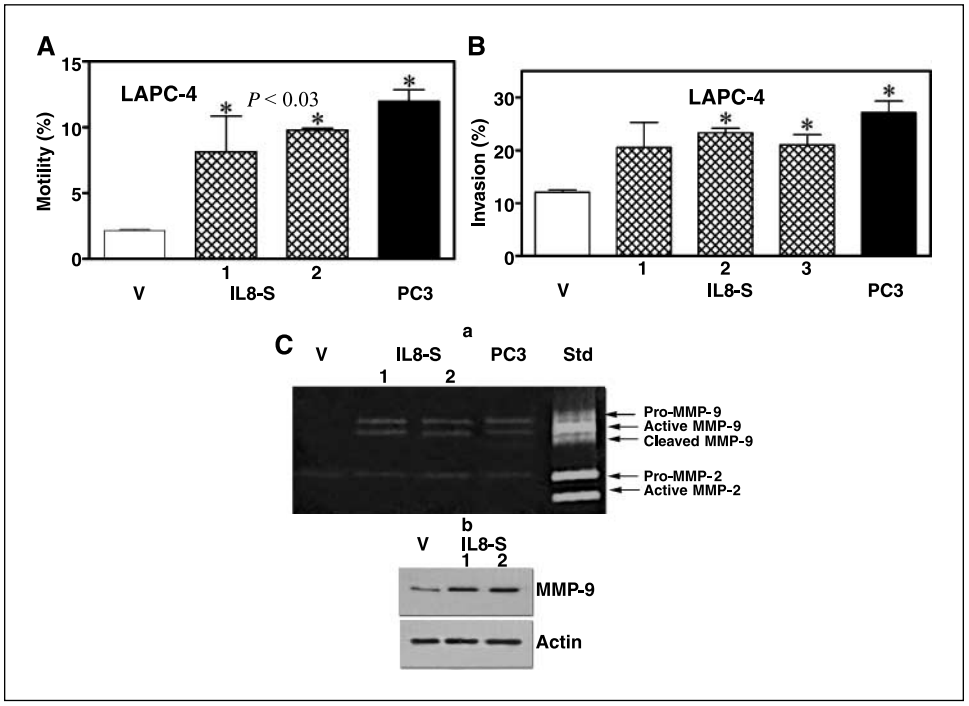

Figure 5. Analysis of chemotactic motility and invasion in LAPC-4 transfectants [IL8-S or vector-only (*V*) transfectants]. *A* and *B,* Motility (*A*) and Matrigel invasion (*B*) assays were done as described in Materials and Methods. *Columns,* mean from three experiments in triplicate; *bars,* SE. *C, a,* LAPC-4 transfectants and PC3 cells were cultured for 72 h and invasion-promoting proteinases were analyzed in the conditioned media by zymography as described in Materials and Methods along with MMP-9 and MMP-2 standards. *b,* immunoblot analysis of MMP-9 in the cell extracts of LAPC-4 transfectants. Loading control, actin. IL8-S, LAPC-4IL8-S; V, vector-only transfectant. *, significant differences in motility and invasive activity between respective vector control and transfectants determined by the *t* test.

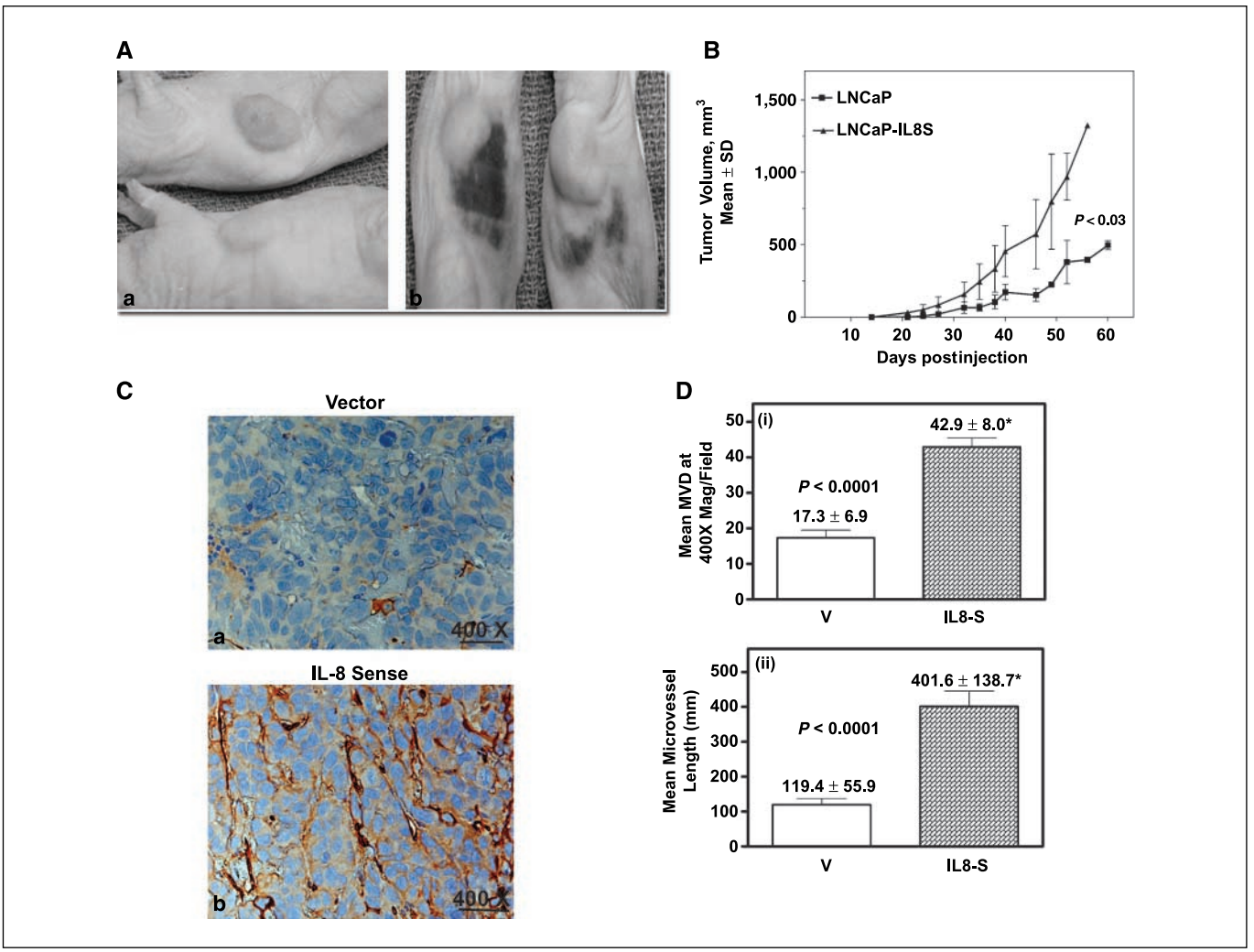

Figure 6. Examination of tumor growth and MVD in xenograft of LNCaP transfectants. Vector and IL8-S transfectants were injected s.c. in athymic male mice (8 animals per group), and tumor volume was measured twice weekly once the tumor became palpable. *A,* vector (*a*) and IL8-S (*b*) tumor-bearing animals euthanized 60 d after injection. Note the necrosis with hemorrhage at or near the tumor site in IL8-S tumor-bearing animals, which is absent in vector tumor-bearing animals. *B,* tumor growth is shown as a function of tumor volume at various days postinjection. *Points,* mean; *bars,* SD. Significance between the growth rates of vector-only versus IL8-S tumors were calculated by comparing the slopes of the growth curve plotted as days postinjection versus log(volume); $P < 0.03$ was obtained by ANOVA (14). *C,* localization of microvessels in vector (*a*) and IL8-S (*b*) tumor specimens. The areas of highest MVD are presented at 400× magnification. *D, i,* MVD in vector and IL8-S transfectants' tumors. Microvessels were counted in 10 random high-power fields representing the highest MVD per tumor specimen. *Columns,* mean; *bars,* SD. *ii,* length of microvessels in vector and IL8-S tumors. The lengths of ≥10 microvessels were measured in five different high-power fields in each specimen (17). *Columns,* mean; *bars,* SD.

IL-8–mediated androgen independence is, in part, due to down-regulation of AR and up-regulation of other survival factors, such as Akt and NFκB; (*d*) IL-8 activates EGFR, src, Akt, and NFκB kinases; this is also the first report to show the potential of IL-8 to activate NFκB; (*e*) IL-8 activation of src and NFκB contributes to drug resistance. The mechanisms underlying IL-8–induced androgen independence are likely to be multiple and are dependent on the cell milieu.

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