Up-Regulation of Tumor Interleukin-8 Expression by Infiltrating Macrophages: Its Correlation with Tumor Angiogenesis and Patient Survival in Non-Small Cell Lung Cancer[1]

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ABSTRACT

Purpose: **To evaluate the interaction between tumor-infiltrating macrophages and cancer cells and its effect on the expression of a potent angiogenic factor, interleukin-8 (IL-8), tumor angiogenesis, and patient outcome in non-small cell lung cancer (NSCLC).**

Experimental Design: **We measured tumor IL-8 mRNA expression (by real-time quantitative reverse transcription-PCR), intratumor microvessel counts, and tumor-infiltrating macrophage density (by immunohistochemical staining) in 35 NSCLC surgical specimens and correlated with the patient's clinical outcome. We then investigated the interaction between macrophages (cell line THP-1) and six different human cancer cell lines (four NSCLCs, one osteosarcoma, and one hepatoma) and its effect on IL-8 mRNA expression using a macrophage/cancer cell coculture system, IL-8 mRNA expression in lung cancer cells, and macrophages being measured separately after coculture in the presence or absence of six anti-inflammatory agents, *i.e.*, pentoxifylline, aspirin, indomethacin, dexamethasone, celecoxib (a selective cyclooxygenase-2 inhibitor), and pyrrolidine dithiocarbamate, a specific nuclear factor κB (NF-κB) inhibitor. NF-κB transcriptional activity and protein levels were measured by reporter gene assay and Western blot.**

Results: **The tumor-infiltrating macrophage density correlated significantly and positively with tumor IL-8 mRNA expression and intratumor microvessel counts and significantly and negatively with patient survival. In addition, after cell–cell interaction in cancer cell:macrophage cocultures, marked IL-8 mRNA expression was induced in lung cancer cells (~270-fold) and, to a lesser degree, in macrophages (4.5-fold). The increase in IL-8 mRNA expression correlated with the *in vitro* metastatic potential of the cancer cells. All six anti-inflammatory agents suppressed induction of IL-8 mRNA expression in lung cancer cells by >90%, four (pentoxifylline, celecoxib, pyrrolidine dithiocarbamate, and dexamethasone) having a dose-dependent effect. NF-κB transcriptional regulation and protein levels were simultaneously increased in the nuclei of cancer cells in macrophage/cancer cell cocultures, this effect also being suppressed by all six anti-inflammatory agents.**

Conclusions: **The interaction between infiltrating macrophages and cancer cells up-regulates IL-8 mRNA expression, especially in the cancer cells; this may contribute greatly to the increased tumor angiogenesis and adverse outcome in NSCLC patients with a high density of tumor-infiltrating macrophages. Anti-inflammatory agents can suppress the induction of IL-8 mRNA expression seen in lung cancer cells after coculture with macrophages, and this suppression is mediated, in part, through the NF-κB pathway.**

INTRODUCTION

Angiogenesis is required for tumor growth, progression, and metastasis (1, 2). Many studies have demonstrated that high intratumor microvessel counts correlate with tumor advancement, systemic metastasis, and prognosis in several human cancers, including melanoma, breast cancers, colon cancers, and lung cancers (3–6). Angiogenesis is a complicated process that involves the degradation of the basement membrane and invasion of the stroma by endothelial cells, which then proliferate, migrate, and become organized into a capillary structure (7). This process is regulated by the local activity of a variety of angiogenic factors, such as IL-8,[4] VEGF, and basic fibroblast growth factor (2, 8, 9).

Previous studies have shown that angiogenic factors can be produced by either the tumor cells or their stroma (10–12).

Received 5/7/02; revised 10/10/02; accepted 10/16/02.
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[1] Supported by NSC 91-3112-P-005-008-Y (Taipei, Taiwan, ROC).
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[4] The abbreviations used are: IL, interleukin; NF-κB, nuclear factor κB; NSCLC, non-small cell lung cancer; FBS, fetal bovine serum; VEGF, vascular endothelial growth factor; RTQ-RT-PCR, real-time quantitative reverse transcription-PCR; IHC, immunohistochemical staining; PDTC, pyrrolidine dithiocarbamate; TBP, TATA box binding protein; TNF, tumor necrosis factor; ATCC, American Type Culture Collection; CT, threshold cycle; COX, cyclooxygenase; CI, confidence interval; NSAID, nonsteroidal anti-inflammatory drug.

Recently, IL-8 was shown to be an important angiogenic factor in NSCLC (13). Coculture of fibroblasts and NSCLC cells also demonstrated that IL-8 mRNA and protein can be induced in both cancer cells and stroma through an indirect interaction (14). The increased IL-8 expression seen in monocytes after coculture with NSCLC cells also indicates that cancer cells can stimulate inflammatory cells to express increased amounts of angiogenic factors (15).

IL-8, a member of the C-X-C chemokine family, originally classified as a neutrophil chemoattractant with inflammatory activity (16), is a potent angiogenic factor in several cancers and is associated with metastasis (17–20). In our previous study, we used RTQ-RT-PCR to demonstrate that IL-8 mRNA expression correlates with tumor progression, angiogenesis, patient survival, and the timing of relapse in NSCLC (21).

Recently, a number of reports have shown that tumor-infiltrating macrophages constitute an important interface between tumor cells and the immune system and that they might influence neoplastic growth and progression in several ways (22–24). Macrophage infiltration was shown recently to correlate with vessel density in ovarian (25), breast (26), and central nervous system (27) malignancies and be associated with the expression of VEGF and epidermal growth factor receptor in breast cancer cells (28). Takanami *et al.* (29) have shown that a high density of tumor-associated macrophages is associated with angiogenesis and an adverse prognosis in lung cancer (adenocarcinoma), but little is known about how macrophages contribute to angiogenesis or about the relationship between the tumor-infiltrating macrophage and cancer cells and how this affects IL-8 expression, angiogenesis, and patient outcome in NSCLC. In this study, we investigated the interaction between infiltrating macrophages and cancer cells and hypothesized that tumor-infiltrating macrophages might interact with cancer cells and influence IL-8 mRNA expression and angiogenesis in NSCLC.

We found that the infiltrating macrophage density correlated significantly and positively with tumor IL-8 mRNA expression and intratumor microvessel counts and significantly and negatively with patient survival. Furthermore, we found that IL-8 mRNA expression in cancer cells was dramatically increased by ~270-fold after interaction with macrophages and that this increase could be suppressed by several anti-inflammatory agents.

MATERIALS AND METHODS

Cell Lines and Clinical Specimens. The human monocyte cell line THP-1 (ATCC TIB202; ATCC, Manassas, VA), NSCLC cell line A549 (ATCC CCL-185), and lung adenocarcinoma cell lines CL1-0, CL1-5, and PC14 (30, 31) were grown in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS (Life Technologies, Inc.). The human osteogenic sarcoma cell line Saos2 and hepatoma cell line HepG2 were maintained in DMEM (Life Technologies, Inc.) containing 10% FBS.

Monocytes were stimulated to differentiate into macrophages by the addition of 3.2×10^{-7} M phorbol myristate acetate (Sigma Chemical Co., St. Louis, MO) for 24 h. All cells were incubated at 37°C in 20% O_2 and 5% CO_2.

Thirty-five consecutive patients who had undergone resection for clinical stage I, II, or IIIA NSCLC (32) at our institute between May and December 1994 were included in the study. The clinicopathologic features of the patients and tissues are shown in Table 1. Paraffin-embedded, formalin-fixed, surgical specimens were collected for histological examination and immunohistochemical studies of IL-8 protein expression, intratumor microvessel counts, and macrophage number. Lung cancer tissue specimens, obtained at surgery and immediately frozen, were used to quantify IL-8 mRNA expression.

Table 1 Summary of clinicopathologic features of patients and tumors

Characteristics	No.
Age: years (mean)	60.3
Sex	
Male	24
Female	11
Histology	
Squamous cell carcinoma	17
Adenocarcinoma	18
Stage[a]	
SI	14
SII	4
SIII	17
T	
T_1	5
T_2	19
T_3	11
N	
N_0	19
N_1	6
N_2	9
N_3	1
C/T and/or R/T[b]	
(+)	10
(−)	25
Infiltrating macrophage (median)	162/×200 field
Microvessel count (median)	115/×200 field
IL-8-mRNA (−ΔCT; median)[c]	9.96
Survival (months; median)	30
Relapse (months; median)	18

[a] Stage, T, and N were determined according to the guidelines of the American Joint Committee on Cancer Staging (32).
[b] C/T and/or R/T, chemotherapy and/or radiotherapy.
[c] IL-8 mRNA level expressed as $-\Delta CT = -[CT_{IL\text{-}8} - CT_{TBP}]$.

IHC for IL-8, Microvessels, and Macrophages. IHC was carried out using a modified avidin-biotin peroxidase complex method (8, 33). IL-8 was stained using mouse anti-IL-8 monoclonal antibody (1:200 dilution; Endogen, Woburn, MA) as the primary antibody and rabbit antimouse Ig/IgG antibody (1:10 dilution; Zymed, San Francisco, CA) as the secondary antibody. The tumor specimen was then counterstained with Mayer's hematoxylin solution. Microvessels were stained using mouse polyclonal anti-CD34 antibody (1:20 dilution; Novocastra, Newcastle, United Kingdom) as the primary antibody. Macrophages were stained using mouse anti-CD68 monoclonal antibody (1:100 dilution; DAKO, Carpinteria, CA) as the primary antibody. Microvessel counts and tumor-infiltrating macrophage counts were determined as described in previous reports (21, 33). The color was developed using diaminobenzidine tetrahydrochloride (Zymed). The detailed procedures are described in our previous study (21).

Macrophage/Cancer Cell Cocultures. (I) Effect of Conditioned Medium on IL-8 mRNA Levels in CL1-5 Cells. Cultured macrophages were detached by exposure to versene (1:5000 dilution; Life Technologies, Inc.), washed with 3 × 15 ml of PBS to avoid any effect of phorbol myristate acetate, then incubated in 20 ml of either RPMI/10% FBS or serum-free media for 24 h, and the culture supernatants were later collected as conditioned medium. The CL1-5 human lung adenocarcinoma cell line was cultured for 24 h in RPMI/10% FBS or serum-free medium containing 0, 10, 20, 30, 40, or 50% of the corresponding serum-containing or serum-free, macrophage-conditioned medium; then total RNA was isolated using RNAzol B (Tel-Test, Friendswood, TX) and used to measure IL-8 mRNA levels (see below).

(II) Effect of Coculture on IL-8 mRNA and Protein Production by Cancer Cell Lines. Several cancer cell lines, seeded in six-well plates, were allowed to grow to confluence for 24 h and then washed twice with serum-free RPMI 1640 and incubated in 2.5 ml of serum-free RPMI. Macrophages (5×10^5 in 2 ml of serum-free RPMI) were seeded into the top chamber of a transwell apparatus (Costar, Cambridge, MA), which was then placed directly on top of the six-well plates containing the cancer cells. For Western blot experiments, cancer cells alone were incubated with conditioned medium as described above. After an overnight incubation, the medium was collected and centrifuged to remove cellular debris, and the supernatants were frozen at −80°C until assayed for IL-8 by ELISA. The cells in the six-well plates were washed twice with PBS and collected so that the cell numbers should be counted. Total RNAs were extracted using 1 ml of RNAzol B to assay IL-8 mRNA levels. The cell number was used to normalize the IL-8 protein expression in ELISA. All experiments were performed in triplicate.

Anti-inflammatory Drug Treatment. The anti-inflammatory drugs pentoxifylline, PDTC, aspirin, indomethacin, and dexamethasone were purchased from Sigma, whereas celecoxib was a gift from Searle (Caguas, Puerto Rico). The designated concentration of anti-inflammatory drug was added to macrophage/cancer cell cocultures or, for Western blot studies, to the macrophage-derived conditioned medium. The viability of drug-treated cells was evaluated using the trypan blue dye exclusion method. After incubation for 24 h, the medium was collected and frozen at −80°C until assayed for IL-8 by ELISA, whereas the cells were harvested, and total RNA was extracted with RNAzol B and used for IL-8 mRNA quantification.

IL-8 mRNA Expression Quantified Using RTQ-RT-PCR. Total RNA was extracted from resected lung cancer tissues, monocytes, macrophages, and lung cancer cell lines/macrophage cocultures using RNAzol B. The primers and probe used for RTQ-RT-PCR of IL-8 have been described in our previous study (21). The TBP was quantified as an internal control using the primers and probe described in a previous study (34). Briefly, each amplification mixture (50 μl) containing 10 ng of total RNA underwent one cycle of the reverse transcription program, followed by 40 cycles of the amplification program. Each assay included a standard curve, a nontemplate control, and total RNA samples, all in triplicate.

The CT is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeds a fixed threshold above the baseline; at a given threshold, a higher CT value indicates a lower starting copy number. The amount of IL-8 cDNA relative to the amount of TBP cDNA was measured as $-\Delta CT = -[CT_{IL\text{-}8} - CT_{TBP}]$. The ratio of IL-8 mRNA copies relative to TBP mRNA copies was defined as $2^{-\Delta CT} \times K$ (K: constant).

Preparation of Nuclear Extracts. The method used was a slight modification of a method described previously (35). Briefly, adherent cells were scraped into 1.5 ml of cold PBS, pelleted by centrifugation, and resuspended in 400 μl of cold hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride]. The cells were swelled on ice for 10 min and then centrifuged at 5,000 × *g* for 5 min at 4°C. The nuclear pellet was resuspended in 80 μl of cold high salt buffer [20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride] and incubated on ice for 20 min, then centrifuged at 17,800 × *g* for 5 min at 4°C. The supernatant containing the nuclear extract was harvested and stored at −80°C.

Western Blot Analysis. Equal amounts of nuclear extract (20 μg of protein) were boiled for 5 min in sample buffer [125 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 20% glycerol, and 0.005% bromphenol blue] and then subjected to PAGE. After transfer to a nitrocellulose membrane (Hybond-C Extra; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), NF-κB was detected using rabbit polyclonal anti-NF-κB primary antibody (1:200; NF-κB p65, c20; Santa Cruz Biotechnology, Santa Cruz, CA), alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody (1:500; Santa Cruz Biotechnology), and CDP-Star chemiluminescent substrate (Tropix, Bedford, MA). β-actin was detected using goat-polyclonal-IgG anti-β-actin primary antibody (Santa Cruz Biotechnology) as the loading control.

Transcriptional Regulation Assay. NF-κB transcriptional activity was determined by reporter gene assay. The CL1-5 cells were seeded into six-well plates with the cell density of 5×10^4 cells/well. The cancer cells were allowed to grow for 24 h and then washed and incubated in 3 ml of serum-free RPMI. The cells were cotransfected with NF-κB-binding domain luciferase (pGL2−4 × AGGGGACTTTCC-luciferase; a gift from Dr. Shuang-En Chuang, National Health Research Institute) and pSV-β-galactosidase constructs (Promega, Madison, WI) by Lipofectamine method (36). The pSV-β-galactosidase construct was used to normalize the transfection efficiency. After 4 h of incubation, transfected cells were washed twice with RPMI 1640 and incubated in 3 ml of RPMI with 10% FBS to recover for 24 h. Then, the transfected CL1-5 cells were cocultured with macrophages in six-well transwells with or without anti-inflammatory drugs at the designated concentration for another 24 h. Cell lysate was harvested to assay the luciferase activity according to the Tropix Luciferase Assay kit (Tropix). All of the experiments included nontransfection and vector alone as negative controls were performed in triplicate and normalized with both transfection efficiency and protein quantity.

Statistical Analysis. All experiments were performed in triplicate and analyzed by ANOVA (Excel, Microsoft; Taipei, Taiwan, Republic of China) for significant differences. Rela-

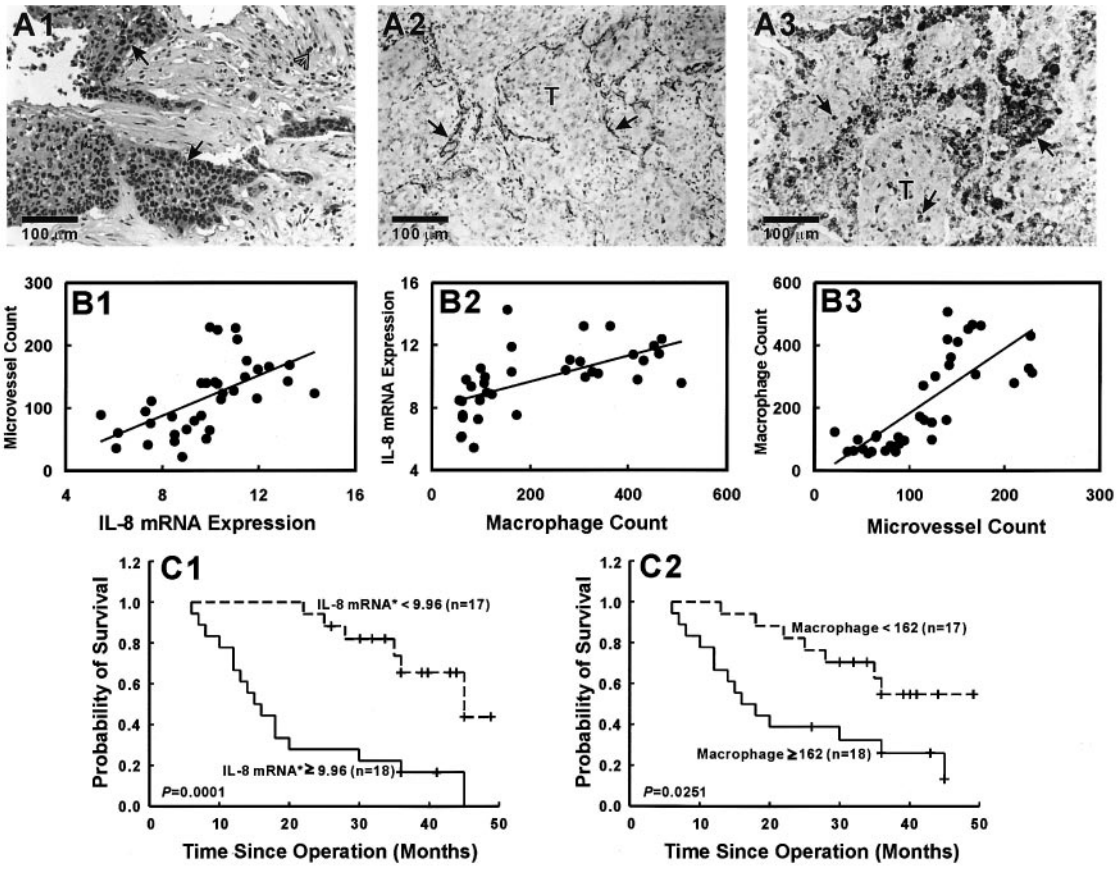

Fig. 1 IL-8 protein, microvessels, and macrophages in NSCLC samples and their interrelationships and correlation with survival. *A*, IHC of IL-8 protein (*A1*), microvessels (*A2*), and macrophages (*A3*). Original magnifications: ×200. Scale bars in each graph: 100 μm. *B*, correlation of IL-8 mRNA expression with microvessel counts ($r = 0.591$, $P < 0.001$; *B1*) or macrophage count ($r = 0.604$, $P < 0.001$; *B2*) and of microvessel counts with macrophage counts ($r = 0.775$, $P < 0.001$; *B3*). *C*, Kaplan-Meier survival plots for NSCLC patients grouped according to IL-8 mRNA expression (*C1*) or tumor-infiltrating macrophage density (*C2*). *Value derived from $-\Delta$CT. Higher IL-8 mRNA levels and higher macrophage counts are associated with lower survival ($P = 0.0001$ and 0.0251, respectively).

tionships between IL-8 mRNA expression, microvessel counts, and macrophage density were analyzed by linear regression. Survival curves were obtained by the Kaplan-Meier method, and the difference in survival in subgroups was analyzed using the Log-rank test (SPSS software). *P*s < 0.05 were considered statistically significant. Where appropriate, the data are presented as the mean ± SD.

RESULTS

Infiltrating Macrophages Are Significantly Correlated with IL-8 Expression, Angiogenesis, and Patients' Survival. To explore the relationships between macrophage density, IL-8 mRNA expression, and microvessel counts, 35 lung cancer tumor specimens were examined by RTQ-RT-PCR and IHC. Representative examples of IHC for IL-8, microvessels, and macrophages are shown in Fig. 1*A1–A3*.

IL-8 protein was seen in the cytoplasm of cancer cells in the tumor specimens and of the infiltrating macrophages (indicated by *arrowhead*; Fig. 1*A1*). Immunostained microvessels appeared as brown linear fragments, with or without an internal lumen (Fig. 1*A2*), whereas the cytoplasm of tumor-infiltrating macrophages was stained brown by anti-CD-68 antibodies (Fig. 1*A3*).

Significant correlations were found between IL-8 mRNA expression and the microvessel count (linear regression, $r = 0.591$, $P < 0.001$; Fig. 1*B1*), IL-8 protein expression and macrophage density (linear regression, $r = 0.604$, $P < 0.001$; Fig. 1*B2*), and the microvessel count and macrophage density (linear regression, $r = 0.775$, $P < 0.001$; Fig. 1*B3*).

We used a median value of 9.96 ($-\Delta$CT) as the cutoff to separate tumors into high ($n = 18$) and low ($n = 17$) IL-8 mRNA-expressing tumors and a median value of 162 (infiltrating macrophage density/×200 field) as the cutoff to separate tumors into those with high ($n = 18$) and low ($n = 17$) infiltrating macrophage counts. Patients with tumors expressing high levels of IL-8 mRNA had a significantly shorter median survival (15 months; 95% CI: 10.8–19.2) than those with tumors expressing low levels of IL-8 mRNA (45 months; 95% CI: 29–61 months; Log-rank test, $P < 0.0001$; Fig. 1*C1*). The median survival for patients with tumors with a high density of tumor-infiltrating macrophages (16 months; 95% CI: 9.8–22.2 months) was also significantly shorter than that for patients

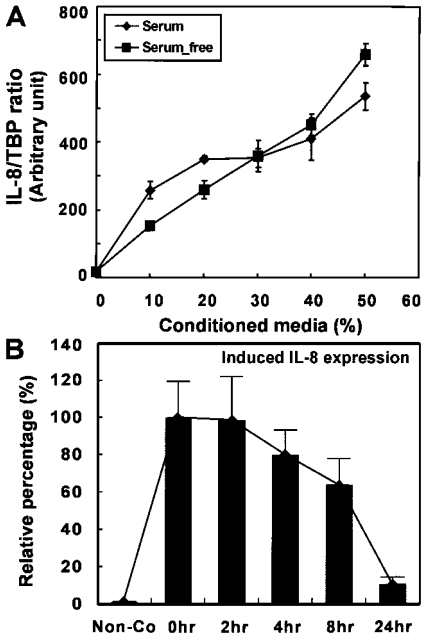

Fig. 2 Effect of macrophage-conditioned medium or serum on IL-8 expression by the lung cancer cell line CL1-5. Cancer cells were incubated in serum or serum-free RPMI media containing different proportions of conditioned media for 24 h. IL-8 mRNA expression was determined by RTQ-RT-PCR. In *A,* there was a highly significant difference in IL-8 mRNA expression at different concentrations of conditioned medium (ANOVA, $\alpha = 0.05$; $P < 0.00001$), but there was no difference in the presence or absence of serum (ANOVA, $\alpha = 0.05$; $P = 0.72$). In *B,* after discontinuation of macrophage cocultures, cells were washed with PBS and then incubated in serum-free media for 24 h. IL-8 expression in cancer cells decreased with time (0, 2, 4, 8, and 24 h); the relative percentages were 100, 98.54, 80.23, 64.2, and 10.65%, respectively. *Non-Co,* noncoculture. All experiments were performed in triplicate.

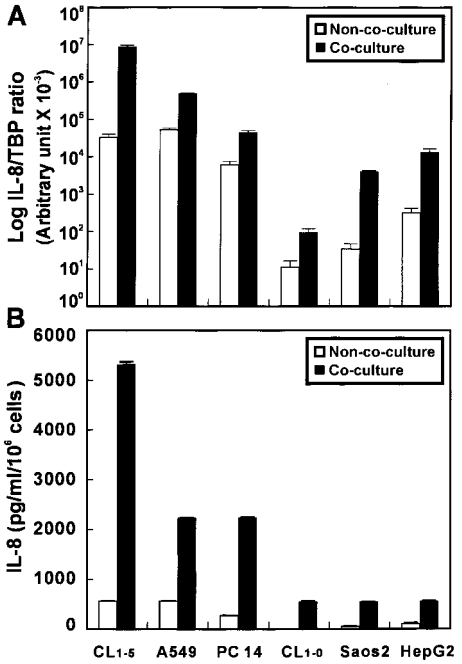

Fig. 3 Induction of IL-8 mRNA expression in cocultured cancer cell lines and protein levels in cocultured media. After macrophages/cancer cell cocultures, cancer cells were harvested to isolate total RNA. Total RNA (10 ng) for each sample was used to determine IL-8 mRNA expression by RTQ-RT-PCR. The cocultured media were saved to perform IL-8 ELISA. In *A,* the *unfilled bars* represent IL-8 mRNA expression of noncocultured cancer cells, and the *filled bars* represent those for cocultured cancer cells. In *B,* higher IL-8 protein is secreted in all cancer cell lines/macrophage-cocultured media (*filled bars*) than noncocultured media (*unfilled bars*). The trend in protein expression is similar to that for the mRNA.

whose tumors had a low density of tumor-infiltrating macrophages (45 months; 95% CI: 33.6–56.4; Log-rank test, $P = 0.0251$; Fig. 1*C2*).

IL-8 mRNA Expression of Cancer Cell Is not Serum Responsible but Soluble Factors in Conditioned Media. Fig. 2*A* shows that IL-8 mRNA, evaluated by RTQ-RT-PCR, increased in CL1-5 cells as the amount of macrophage-conditioned medium added was increased and that this occurred in both serum-containing and -free medium. Using 50% conditioned medium, IL-8 mRNA levels were increased 36-fold (18 ± 2.5 and 660 ± 32.6 in the absence or presence of conditioned medium, respectively) in serum-free RPMI and 30-fold (18 ± 6.1 and 535 ± 39.4) in serum-containing RPMI. The ANOVA test showed no significant difference between the results in serum-containing and -free medium ($\alpha = 0.05$, $P = 0.72$) but a highly significant difference between the results obtained using different amounts of conditioned medium ($\alpha = 0.05$, $P < 0.00001$). Fig. 2*B* shows that, after discontinuance of macrophage cocultures, IL-8 expression in the cancer cells decreased with time but persisted for ≥24 h.

Cancer Cell:Macrophage Cocultures Elevate IL-8 mRNA Expression in Cancer Cells. After coculture of macrophages and six cancer cell lines, total RNA was extracted from the cancer cells, and IL-8 mRNA expression was measured. As shown in Fig. 3*A,* coculture resulted in a 270-, 7.3-, or 8.6-fold increase in IL-8 mRNA expression in the human lung adenocarcinoma cell lines CL1-5, PC14, and CL1-0, a 9.6-fold increase in the A549 NSCLC cell line, a 118-fold increase in the Saos2 osteogenic sarcoma cell line, and a 41.8-fold increase in the HepG2 hepatoma cell line. Expression of IL-8 mRNA was higher than that of the TBP internal control in CL1-5, A549, and PC14 cells with or without macrophage coculture, whereas in CL1-0, Saos2, and HepG2 cells, IL-8 expression was only higher than TBP expression after macrophage coculture.

ELISA analyses showed that IL-8 protein secretion was higher in all cancer cell lines/macrophage cocultures than in the cancer cell lines alone (Fig. 3*B*). IL-8 protein levels were increased between 3.3- and 9.4-fold, and the trend in protein expression was similar to that for IL-8 mRNA (Fig. 3*A*).

Macrophage/Cancer Cell Cocultures Elevate IL-8 mRNA Expression in Macrophages. IL-8 mRNA levels were measured in monocytes, nonsensitized macrophages, and macrophages after coculture with cancer cells. As shown in Fig. 4, IL-8 mRNA levels in monocytes were very low (IL-8:TBP ratio $= 1.24 \pm 0.055$), whereas levels in monocyte-derived macrophages were 286 times higher, and coculture with three lung cancer cell lines, CL1-5, A549, and PC14, resulted in an

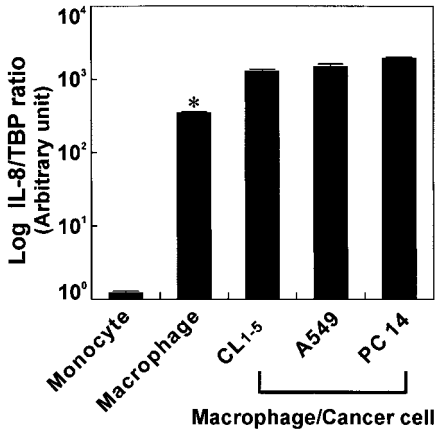

Fig. 4 Induction of IL-8 mRNA expression in cocultured macrophages. After cocultures for 24 h, macrophages were harvested to isolate total RNA. Total RNA (10 ng) for each sample was used to determine IL-8 mRNA expression by RTQ-RT-PCR. Macrophages cocultured with three lung cancer cell lines show increased IL-8 mRNA expression in cocultured macrophages compared with nonsensitized macrophages or monocytes. $*P < 0.01$ compared with monocytes alone or macrophages cocultured with cell line CL1-5, A549, or PC14.

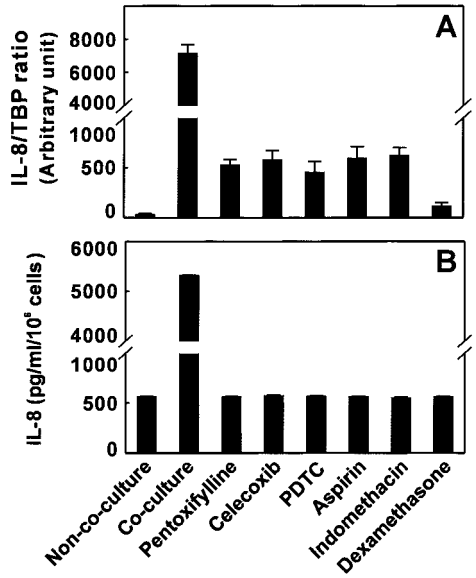

Fig. 5 Anti-inflammatory agents suppress IL-8 mRNA expression in cocultured CL1-5 cells and IL-8 protein secretion in cocultured media. Cancer cells were cocultured with macrophages, together with 10 mM pentoxifylline, 10 μM celecoxib, 50 μM PDTC, 100 μM aspirin, 1 μM indomethacin, and 1 μM dexamethasone, respectively. The average cell viability of all drug treatment is 94.6 ± 2.66%. In *A*, IL-8 mRNA expressions of cocultured cancer cells were significantly reduced after treatment with each anti-inflammatory drug. All experiments were performed in triplicate. In *B*, IL-8 protein secretion was elevated ≤9.4-fold in CL1-5 cells/macrophage-cocultured media and suppressed to a basal level in cocultured media containing each of the six inflammatory agents. The trend in protein expression is similar to that for the mRNA.

average 4.5-fold increase in macrophage IL-8 mRNA levels. The results shown in Figs. 3 and 4 show that the interaction of macrophages and cancer cells enhances IL-8 mRNA expression in both sets of cells.

Anti-inflammatory Drugs Can Dose Dependently Suppress IL-8 Expression of Coculturing CL1-5 after Coculture with Macrophages. Because inflammatory cells are involved in the increased production of angiogenic factors by cancer cells, anti-inflammatory agents should have the potential to block IL-8-inducing pathways. On the basis of cell viability analyses, 10 mM pentoxifylline (92.8% cell viability), 10 μM celecoxib (91% cell viability), 50 μM PDTC (94.1% cell viability), 100 μM aspirin (96.3% cell viability), 1 μM indomethacin (95% cell viability), or 1 μM dexamethasone (98.6% cell viability) was added to CL1-5 cell/macrophage cocultures for 24-h incubation, resulting in a significant reduction in IL-8 mRNA expression in the CL1-5 cells to 7.4, 8.13, 6.36, 8.33, 8.75, or 1.64%, respectively, of the level in the absence of anti-inflammatory agents (Fig. 5*A*).

In addition, when the same concentrations of anti-inflammatory agents were added to CL1-5 cells/macrophage cocultures, ELISA analysis showed that the induction of IL-8 protein secretion fell to the levels seen in CL1-5 cells in the absence of macrophages (Fig. 5*B*). When tested under conditions of ≥92.8% cell viability, 0.01, 0.1, 1, and 10 mM pentoxifylline resulted in a dose-dependent suppression of IL-8 mRNA expression, the relative percentage expression being 35.5, 19.3, 10.4, and 8.1%, respectively (Fig. 6*A*). Similar results were obtained using celecoxib, PDTC, and dexamethasone (Fig. 6*B–D*, respectively).

IL-8 Expression in Cancer Cells after Cocultured with Macrophages Is Partly Regulated through the NF-κB Pathway. Fig. 7*A* shows that NF-κB protein levels in CL1-5, A549, and PC14 cancer cells incubated with 100% conditioned medium increased in parallel with the increase in IL-8 mRNA expression shown in Fig. 3*A*. Fig. 7*B* shows that NF-κB protein levels in CL1-5 cells grown in conditioned medium were ~20-fold higher than in those grown in the absence of conditioned medium *(Lanes 2* and *1*, respectively). In addition, to understand the regulatory pathway of IL-8 expression in indirect cell–cell interaction, the six anti-inflammatory agents described above added to the conditioned medium and their effects on NF-κB protein levels were examined. In the Western blots shown in Fig. 7*B*, a comparison of *Lanes 3–8* (pentoxifylline, celecoxib, PDTC, aspirin, indomethacin, and dexamethasone treatment, respectively) with *Lane 2* shows that NF-κB protein levels were significantly decreased in CL1-5 grown in conditioned medium in the presence of the six anti-inflammatory drugs, especially those treated with PDTC (reduced by 90.5%) or dexamethasone (reduced by 96.1%).

The NF-κB transcriptional activity was assessed by luciferase reporter gene assay. As shown in Fig. 8, the luciferase activity in CL1-5 cells after coculture with macrophage was increased 40-fold. The addition of anti-inflammatory agents could significantly suppress the NF-κB transcriptional activity. The mock transfectant and nontransfected control cells revealed no response in luciferase activity assay.

On the basis of these results, we suggest that the increase in IL-8 expression by cancer cells induced by macrophage-conditioned medium is, at least in the case of CL1-5, regulated in part through the NF-κB pathway.

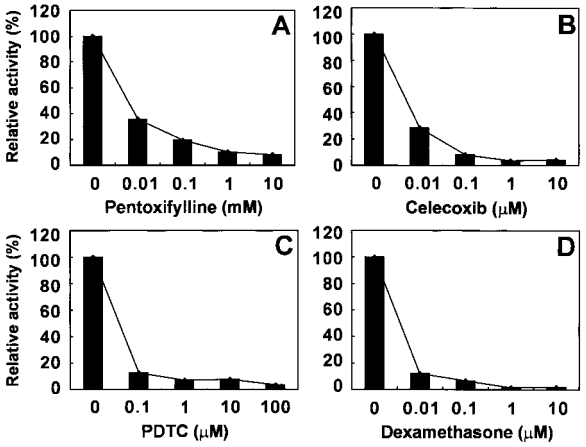

Fig. 6 Dose-dependent effect of anti-inflammatory agents in suppressing IL-8 mRNA expression in cocultured CL1-5 cells. Cocultures were performed in the presence or absence of various concentrations of pentoxifylline (*A*), celecoxib (*B*), PDTC (*C*), or dexamethasone (*D*). The least cell viability was 92.8% at all of the designated concentrations. After incubation for 24 h, the cancer cells were harvested, and total RNA was extracted and then used for IL-8 mRNA quantification. The serial dilutions of pentoxifylline (0, 0.1, 1, and 10 mM) revealed the dose-dependent suppression of IL-8 mRNA expression. Similar results were obtained using celecoxib, PDTC, and dexamethasone.

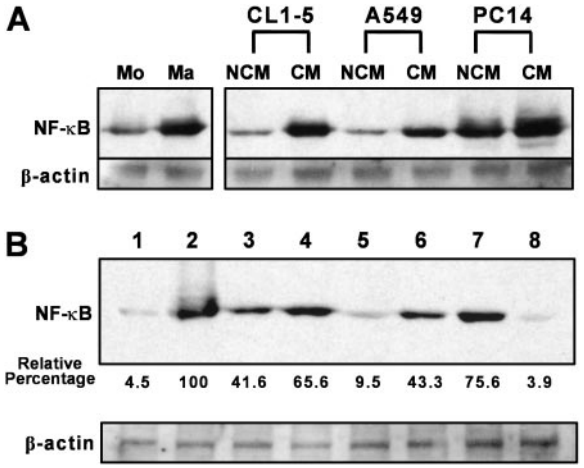

Fig. 7 Western blotting analysis of NF-κB protein levels in cancer cells grown in the presence or absence of macrophage-conditioned medium and the effect of anti-inflammatory drugs. *A*, monocytes (*Mo*) or macrophages (*Ma*) alone or cell lines CL1-5, A549, or PC14 cultured without conditioned medium (*NCM*) or with conditioned medium (*CM*). *B*, *Lane 1*, CL1-5 cells without conditioned medium; *Lanes 2–8*, CL1-5 cells with conditioned medium in the absence of anti-inflammatory agents (*Lane 2*) or in the presence of 10 mM pentoxifylline (*Lane 3*), 10 μM celecoxib (*Lane 4*), 50 μM PDTC (*Lane 5*), 100 μM aspirin (*Lane 6*), 1 μM indomethacin (*Lane 7*), or 1 μM dexamethasone (*Lane 8*). The number below each lane represents the relative percentage of NF-κB protein level compared with *Lane 2*. β-actin was shown as the loading control.

DISCUSSION

Our previous study in lung cancer patients confirmed that IL-8 mRNA expression in tumor specimens correlates with tumor progression, tumor angiogenesis, patient survival, and occurrence of tumor metastasis (21). In this study, using RTQ-RT-PCR and immunohistochemistry, we further demonstrated that, in lung cancer specimens, the density of tumor-infiltrating macrophages within the tumor correlated significantly with IL-8 mRNA levels, microvessel counts, and patient survival. In addition, cell culture experiments showed that tumor IL-8 mRNA expression was increased 7.3–270-fold in lung cancer cells after cocultured with macrophages and that this up-regulation of IL-8 mRNA expression persisted for ≥24 h after discontinuance of macrophage cocultures. Anti-inflammatory agents could suppress >93% of the IL-8 expression induced in CL1-5 cells by coculture with macrophages, and a specific NF-κB inhibitor, PDTC, resulted in 94% suppression. The interaction between the cancer cells and macrophages was, in part, mediated through soluble factors that involved the NF-κB pathway.

Tumor-infiltrating macrophages can be activated in malignant tumors, and this may contribute to tumor angiogenesis (37, 38). There is also a significant correlation between the number of infiltrating macrophages and angiogenesis (39, 40). Tumor-associated, macrophage-derived angiogenic factors include VEGF, basic fibroblast growth factor, platelet-derived endothelial cell growth factor, TNF-α, and IL-8 (28, 41). The study of Liss *et al.* (42) supports the idea that, in addition to the infiltrating macrophages, tumor cells themselves may be activated by the macrophages and secrete angiogenic factors, which might contribute to tumor angiogenesis in head-and-neck squamous cell carcinomas. In this study, we confirmed this hypothesis by showing that IL-8 expression in cancer cells can be increased ≤270-fold after interaction with macrophages.

The induced expression of angiogenic factors seen during indirect cell–cell interaction between tumor and stroma cells (14, 15) indicated that secreted factors, present in conditioned medium, mediate cell–cell communication. Our data also demonstrated that the addition of macrophage-derived conditioned medium enhanced IL-8 mRNA expression in CL1-5 lung adenocarcinoma cells and that this effect was dose dependent and serum independent. However, it is still unknown how many other factors are involved in this regulatory process.

The use of a coculture system in which the cells are physically separated showed that IL-8 mRNA expression was synergistically increased both in macrophages and cancer cells after coculture. IHC showed that the cancer cell:macrophage ratio in sections was ~11.2 ± 5.67 and that IL-8 protein was distributed throughout the entire tumor section, whereas the RTQ-RT-PCR results showed that IL-8 mRNA expression was dramatically induced in macrophage-cocultured cancer cells. These results show that macrophages seem to play an important initiator role in the regulatory pathway of IL-8 expression in cancer cells. In contrast to the results of White *et al.* (15), who reported that macrophages were the main source of IL-8 in cancer cell:macrophage cocultures, our experiments showed that sensitized cancer cells were the major source of angiogenic factors in cocultures and tumor specimens.

The amplification and propagation of IL-8 expression in cocultures were seen not only with lung cancer cells but also with other tumor cell types (osteosarcoma and hepatoma), suggesting that increased IL-8 mRNA expression may be a common feature of the cancer cell:macrophage interaction. Although the pulmonary cancer cell lines CL1-0 and CL1-5 were derived from the same

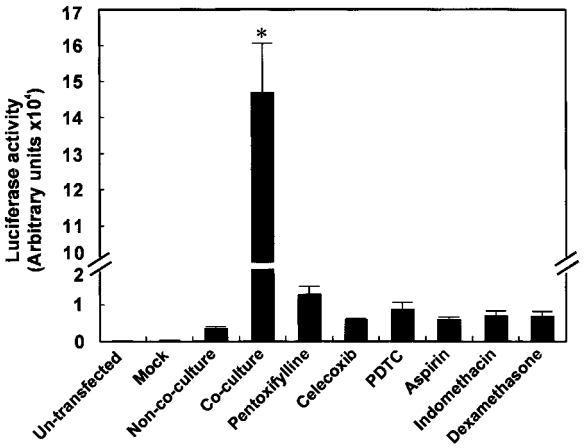

Fig. 8 Reporter gene assay of NF-κB transcriptional activity. CL1-5 cells were transfected with NF-κB-binding domain luciferase construct and then cocultured with macrophages for 24 h with or without anti-inflammatory drugs (10 mM pentoxifylline, 10 μM celecoxib, 50 μM PDTC, 100 μM aspirin, 1 μM indomethacin, and 1 μM dexamethasone, respectively). Luciferase activity assay was performed, and the results showed that it was significantly decreased in cocultures with drugs. *$\alpha = 0.05$, $P < 0.00005$ in average compared with cocultures with drugs treatment.

parental cells, they possess different invasive/metastatic potential *in vitro* and *in vivo*, CL1-5 having a greater potential than CL1-0 (30). Our results therefore show that IL-8 expression was increased to a lesser degree in cells with low metastatic capacity (CL1-0) than in cells with a higher metastatic capacity (CL1-5).

All six anti-inflammatory agents tested significantly inhibited IL-8 mRNA expression in CL1-5 human lung adenocarcinoma cells cocultured with macrophages. All four tested showed a dose-dependent effect in inhibiting IL-8 expression in lung cancer cell:macrophage cocultures. Although the suppressive effects of these drugs were very similar (except for dexamethasone, which was much more effective), their possible mechanisms of action are quite different. Pentoxifylline is a phosphodiesterase inhibitor, which can inhibit the surface expression of intercellular adhesion molecule 1 and the production of IL-8 by cytokine-activated A549 cells (43). It also inhibits protein kinase C-dependent activation of NF-κB and prevents hypoxia-induced expression of VEGF (44). Celecoxib, a COX-2 inhibitor, is an NSAID and can induce apoptosis in prostate cancer cells (45). PDTC can inhibit NF-κB activation and the expression of proinflammatory genes, such as the TNF-α and intercellular adhesion molecule-1 expression induced by lipopolysaccharide (46). Aspirin, a nonsteroidal COX inhibitor, can block TNF-α-induced IL-8 expression and inhibit NF-κB activation (47). Indomethacin, a nonselective COX inhibitor, cannot inhibit transforming growth factor β1-induced IL-8 release but does inhibit transforming growth factor-β1-induced prostaglandin E_2 release (48). Dexamethasone, a glucocorticoid analogue, suppresses IL-8 expression by inhibiting NF-κB activation in a human glioblastoma cell line (49) or through the post-transcriptional mechanism in airway epithelial cells (50).

The reporter gene assay of the NF-κB-binding domain revealed that not only the NF-κB protein level was increased in cancer cell:macrophage cocultures but also the transcriptional activity. Our results also demonstrated that the inhibitory effect of the anti-inflammatory drugs was, for the most part, mediated through the NF-κB pathway. Nevertheless, the complete mechanisms of IL-8 gene suppression by these anti-inflammatory drugs in CL1-5 cells remain to be further investigated.

Taken together, these results show that, because macrophages are involved in the production of angiogenic factors by cancer cells, anti-inflammatory agents have the potential to impede the pathway of IL-8 induction. This study also supports findings from previous studies that some NSAIDs, such as aspirin, can reduce the risk of developing colorectal and breast cancers (51, 52). One of the mechanisms by which NSAIDs exert this effect might be by suppressing the expression of inducible inflammatory cytokines, such as IL-8, by cancer cells and the subsequent angiogenesis.

ACKNOWLEDGMENTS

We thank Dr. Wei-Shiung Yang for kindly providing cell lines.

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