Bone-derived SDF-1 stimulates IL-6 release via CXCR4, ERK and NF-κB pathways and promotes osteoclastogenesis in human oral cancer cells

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Oral squamous cell carcinoma (SCC) has a striking tendency to invade to bone. The chemokine stromal cell-derived factor-1 (SDF-1) is constitutively secreted by osteoblasts and plays a key role in homing of hematopoietic cells to the bone marrow. Interleukin (IL)-6 plays an important role in osteoclastogenesis. Herein, we found that SDF-1α increased the secretion of IL-6 in cultured human SCC cells, as shown by reverse transcriptase–polymerase chain reaction and enzyme-linked immunosorbent assay. SDF-1α also increased the surface expression of chemokine receptor 4 (CXCR4) in SCC cells. CXCR4-neutralizing antibody, CXCR4-specific inhibitor (AMD3100) or small interfering RNA against CXCR4 inhibited SDF-1α-induced increase IL-6 production. The transcriptional regulation of IL-6 by SDF-1α was mediated by phosphorylation of extracellular signal-regulated kinases (ERKs) and activation of the nuclear factor-kappa B (NF-κB) components p65 and p50. The binding of p65 and p50 to the NF-κB element on the IL-6 promoter was enhanced by SDF-1α. In addition, IL-6 antibody antagonized the SCC-conditioned medium-increased osteoclastogenesis. These results suggested that SDF-1α from osteoblasts could induce release of IL-6 in human SCC cells via activation of CXCR4, ERK and NF-κB pathway and thereby promote osteoclastogenesis.

Introduction

Oral squamous cell carcinoma (SCC) represents 1–2% of all human malignancies. They are characterized by a high degree of local invasiveness and a high rate of metastasis to cervical lymph nodes, but a low rate of metastasis to distant organs. The invasion of oral SCC into maxillary and mandibular bone is a common clinical problem. The process of invasion consists of well-linked multiple tumor–host interactions. Previous reports suggest that bone destruction in carcinoma invasion and metastasis is mediated by osteoclasts rather than by carcinoma cells directly (1–3).

Interleukin (IL)-6, originally identified as a T-cell-derived cytokine that induces final maturation of B cells into antibody-producing cells

Abbreviations: AP-1, activator protein-1; CXCR4, chemokine receptor 4; ERK, extracellular signal-regulated kinase; IL, interleukin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMP-9, matrix metalloproteinase; mRNA, messenger RNA; NF-κB, nuclear factor-kappa B; ODN, oligonucleotide; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDTC, pyrrolidine dithiocarbamate; RANKL, receptor activator of nuclear factor-kappa B ligand; SCC, squamous cell carcinoma; SDF, stromal cell-derived factor; siCXCR4, small interfering RNA against CXCR4; siRNA, small interfering RNA; TRAP, artrate-resistant acid phosphatase.

(4), exhibits multiple biological activities that differ widely among various types of tissues and cells. Many investigators have reported that IL-6 can enhance or inhibit the proliferation of carcinoma cells (5–9) and that a variety of malignant tumors, including SCCs and adenocarcinomas, have been shown to contain or synthesize IL-6, and autocrine growth stimulation has been suggested as the possible mechanism for the action of IL-6 (10–12). Furthermore, IL-6 also has unique and important effects on bone cells (13). It increases the formation of cells with osteoclast characteristics that have the capacity to resorb bone (14,15). It has also been reported that neutralizing antibody against human IL-6 reversed hypercalcemia associated with human squamous carcinoma by inhibiting osteoclastic bone resorption (16).

Chemokines are structurally related, small (8–14 kDa) polypeptide signaling molecules, which bind to and activate a family of seven-transmembrane G-protein-coupled receptors, the chemokine receptors (17,18). Chemokines are expressed by many tumor types and can promote mitosis and modulate apoptosis, survival and angiogenesis (19,20). Interaction between the chemokine receptor CXCR4 and its ligand, stromal cell-derived factor 1α (SDF-1α or CXCL12), has been found to play an important role in tumorigenicity, proliferation, metastasis and angiogenesis in many cancers, such as lung cancer, breast cancer, melanoma, glioblastoma, pancreatic cancer, cholangiocarcinoma and basal cell carcinoma cells (21–24). Although the mechanisms underlying SDF-1α/CXCR4-mediated tumor invasion have been studied in some cancers (21–24), the role of SDF-1α/CXCR4 in the process of SCC cells invasion to bone remains largely unknown.

Bone is a common site of cancer metastasis. Several tumors show a particular predilection for metastasis to bone, including breast, prostate and lung cancers. Bone-derived growth factor and chemokines also play central roles as trophic factors that attract breast and prostate cancer cells to bone tissue (25). It has been reported that the chemokine IL-6 is a potent and direct activator of osteoclastic differentiation and bone resorption (25). The SDF-1, constitutively secreted by human osteoblast, has been shown to have a key role in the homing of hematopoietic cells to marrow (26). We hypothesized that osteoblast-derived SDF-1 could be capable of regulating IL-6 levels and promoting osteoclastogenesis in SCC cells. The results show that osteoblasts-derived SDF-1 activates CXCR4 receptor and results in the activation of extracellular signal-regulated kinase (ERK)/IκB kinase αβ (IKKαβ) and nuclear factor-kappa B (NF-κB), leading to upregulation of IL-6 expression and promoting osteoclastogenesis.

Materials and methods

Materials

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for IκBα, p-IκBα, IKKα/β, p65, p50, p-ERK, p-p38, p-JNK, p-Akt, ERK, p38, c-Jun N-terminal kinase (JNK) and Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody specific for IKKα/β phosphorylated at $Ser^{180/181}$ and p65 phosphorylated at Ser^{276} was purchased from Cell Signaling and Neuroscience (Danvers, MA). Pyrrolidine dithiocarbamate (PDTC), L-1-tosylamido-2-phenylenylethyl chloromethyl ketone, PD98059, SB203580, SP600125 and Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol-2-[(R)-2-O-methyl-3-O-octadecylcarbonate]) were obtained from Calbiochem (San Diego, CA). Rabbit polyclonal antibody specific for CXCR4, IL-6, IL-1, transforming growth factor-β and receptor activator of nuclear factor-kappa B ligand (RANKL)-Fc were purchased from R&D Systems (Minneapolis, MN). The

NF-κB inhibitor peptide [sequence contains the nuclear localization sequence (residues 360–369) of the transcription factor NF-κB p50 linked to a peptide cell-permeabilization sequence] was purchased from BIOMOL (Butler Pike, PA). The recombinant human SDF-1α was purchased from PeproTech (Rocky Hill, NJ). IL-6 enzyme immunoassay kit was purchased from Cayman Chemical (Ann Arbor, MI). The NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA). The human full-length CXCR4 was provided by Dr Jun Komano (National Institute of Infectious Diseases, Japan). The p38 dominant-negative mutant was provided by Dr J. Han (South-western Medical Center, Dallas, TX). The JNK dominant-negative mutant was provided by Dr M. Karin (University of California, San Diego, CA). The ERK2 dominant-negative mutant was provided by Dr M. Cobb (South-Western Medical Center). The IKKα−KM(K44A) and IKKβ−KM(K44A) mutants were gifts from Dr H. Nakano (Juntendo University, Tokyo, Japan) (27). The Akt (Akt K179A) mutant was a gift from Dr R. H. Chen (Institute of Molecular Medicine, National Taiwan University, Taipei, Taiwan). pSV-β-galactosidase vector, luciferase assay kit, was purchased from Promega (Madison, WI). All other chemicals were obtained from Sigma-Aldrich (St Louis, MO).

Cell culture

The human oral SCC cell lines (HSC3, SCC4, SCC9) and human osteosarcoma cell line (MG-63) were obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in Dulbecco's modified Eagle's medium that was supplemented with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C with 5% CO_2.

Forty-eight hour-conditioned medium (containing serum) from SCC4 cells were collected, diluted 50% in Dulbecco's modified Eagle's medium and added to cultures of human peripheral blood mononuclear cells (PBMCs) to induce osteoclastogenesis (28).

Isolation and culture of PBMCs and differentiation to osteoclasts

Peripheral blood was collected from healthy donors with heparin anticoagulant, in the presence of 200 ng/ml RANK-Fc, to minimize any priming of osteoclast progenitors by endogenous RANKL as described (28). Blood was diluted in sterile phosphate-buffered saline (PBS) (1:1) in a sterile hood. The blood-PBS solution was slowly layered over Accu-Prep solution (Accurate Chemical and Scientific, Westbury, NY) and then centrifuged at 400*g* in swing buckets for 30 min at 21°C. The PBMC layer was collected and washed in five to six volumes of PBS, isolated by centrifugation at 140*g* and resuspended in α-minimum essential medium containing 10% fetal bovine serum. Cells were counted with a hemocytometer and plated in 48-well tissue culture plates at a concentration of 0.5 million cells in 0.5 ml volume per well. Macrophage colony-stimulating factor-1 (25 ng/ml) was added to all groups. RANKL (25 ng/ml) was used as a positive control. Cultures were maintained at 37°C and half-feeds were done thrice per week and terminated on day 10. Medium was aspirated and the cells were fixed with 10% formalin. Tartrate-resistant acid phosphatase (TRAP) staining was done (Sigma) for quantitation of TRAP-positive multinucleated cells. TRAP-positive cells with more than three nuclei were counted as osteoclasts in the entire well with four wells per treatment. Cell counts were averaged and the results were expressed as the number of TRAP-positive multinucleated cells per well per treatment group (28).

Measurements of IL-6 production

Human SCC cells were cultured in 24-well culture plates. After reaching confluence, cells were treated with SDF-1α and incubated in a humidified incubator at 37°C for 24 h. For examination of the downstream signaling pathways involved in SDF-1α treatment, cells were pretreated with various inhibitors for 30 min before SDF-1α (100 ng/ml) administration. After incubation, the medium was removed and stored at −80°C until assay. IL-6 in the medium was assayed using IL-6 enzyme immunoassay kits, according to the procedure described by the manufacturer.

Messenger RNA analysis by reverse transcriptase–polymerase chain reaction

Total RNA was extracted from SCC cells using a TRIzol kit (MdBio, Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using oligo(dT) primer, then amplified for 33 cycles using two oligonucleotide (ODN) primers—IL-6: AAATGCCAGCCTGCTGACGAAG and AACAACAATCTGAGGTGCCC-ATGCTAC; CXCR4: AATCTTCCTGCCCACCATCT and GACGCCAACAT AGACCACCT; GAPDH: AAGCCCATCACCATCTTCCAG and AGGGGC-CATCCACAGTCTTCT.

Each polymerase chain reaction (PCR) cycle was carried out for 30 s at 94°C, 30 s at 55°C and 1 min at 68°C.

PCR products were then separated electrophoretically in a 2% agarose DNA gel and stained with ethidium bromide.

Western blot analysis

The cellular lysates were prepared as described previously (29). Proteins were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to Immobilon polyvinyl difluoride membranes. The blots were blocked with 4% bovine serum albumin for 1 h at room temperature and probed with rabbit anti-human antibodies against IκBα, IKKαβ, p65 or p50 (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Flow cytometric analysis

Human SCC cells were plated in six-well dishes. The cells were then washed with PBS and detached with trypsin at 37°C. Cells were fixed for 10 min in PBS containing 1% paraformaldehyde. After rinsing in PBS, the cells were incubated with rabbit anti-human antibody against CXCR4 (1:100) for 1 h at 4°C. Cells were then washed again and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary IgG (1:150; Leinco Tec., Inc., St Louis, MO) for 45 min and analyzed by flow cytometry using FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA) (30).

Transfection and reporter gene assay

Human SCC cells were cotransfected with 0.8 μg κB-luciferase plasmid and 0.4 μg β-galactosidase expression vector. Cells were grown to 80% confluence in 12-well plates and transfected the following day by Lipofectamine 2000 (Invitrogen, Carlsbad, CA). DNA and Lipofectamine 2000 were premixed for 20 min and then applied to the cells. After 24 h transfection, the cells were incubated with the indicated agents. After further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega) was added to each well, and cells were scraped from the dishes. The supernatant was collected after centrifugation at 13 000 r.p.m. for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector.

Synthesis of NF-κB and AP-1 decoy ODNs

We used a phosphorothioate double-stranded decoy ODN carrying the NF-κB/Rel-consensus sequence 5′-CCTTGAA GGGATTTCCCTCC-3′/3′-GGAAC-TTCCCTAAAGGGAGG-5′. The activator protein-1 (AP-1) decoy ODN sequence was 5′-TGTCTGACTCATGTC-3′/3′-ACAGACTGAGTACAG-5′. The mutated (scrambled) form 5′-TTGCCGTACCTGACTTAGCC-3′/3′-AACGGCATGGACTGAATCGG-5′ was used as a control. ODN (5 μM) was mixed with Lipofectamine 2000 (10 μg/ml) for 25 min at room temperature, and the mixture was added to cells in serum-free medium. After 24 h of transient transfection, the cells were used for the following experiments (31).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation analysis was performed as described previously (32). DNA immunoprecipitated by anti-p65 or anti-p50 antibody was purified. The DNA was then extracted with phenol–chloroform. The purified DNA pellet was subjected to PCR. PCR products were then resolved by 1.5% agarose gel electrophoresis and visualized by UV.

The primers 5′-CAAGACATGCCAAAGTGCTG-3′ and 5′-TTGAGACT-CATGGGAAAATCC-3′ were utilized to amplify across the human IL-6 promoter region (−288 to −39) (33).

Statistics

The values given are means ± SEM. The significance of difference between the experimental groups and controls was assessed by Student's *t*-test. The difference was significant if the *P* value was <0.05.

Results

SDF-1 induces IL-6 production in human oral cancer cells

SDF-1 is a powerful chemoattractant cytokine that stimulates directional migration of hematopoietic and non-hematopoietic cells. We hypothesized that osteoblast-derived SDF-1 could be capable of regulating IL-6 levels and promoting osteoclastogenesis in SCC cells. Figure 1A shows that SDF-1α induced the expression of IL-6 messenger RNA (mRNA) and protein levels in SCC cells (HSC3,
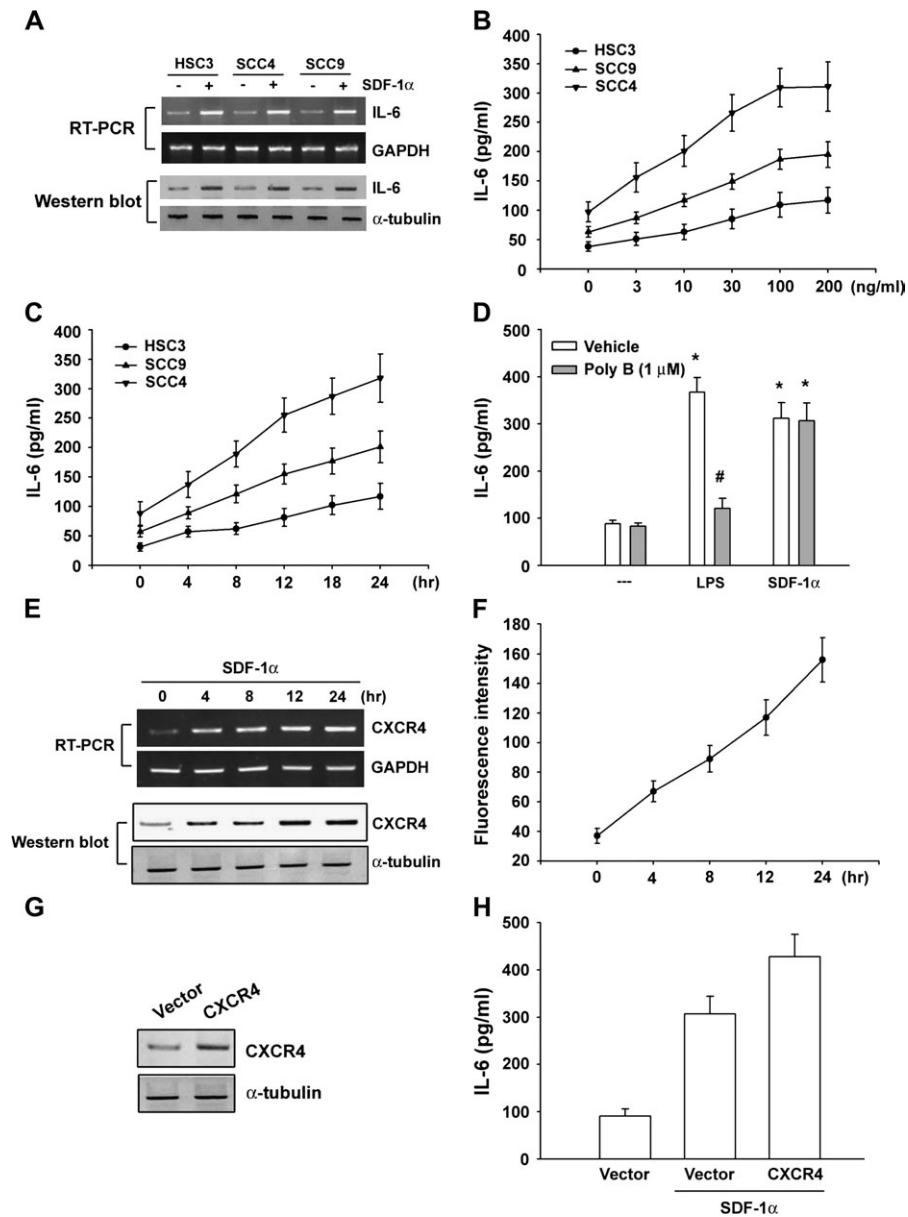
Fig. 1. SDF-1α induced IL-6 production in human SCC cells. (**A**) Human SCC cells (HSC3, SCC4, SCC9) were incubated with SDF-1α (100 ng/ml); the mRNA or protein level of IL-6 were determined by using reverse transcriptase–polymerase chain reaction (RT–PCR) or western blot analysis, respectively. SCC cells were incubated with various concentrations of SDF-1α for 24 h (**B**) or with SDF-1α (100 ng/ml) for 4, 8, 12, 18 or 24 h (**C**). Media were collected to measure IL-6. Results of four independent experiments performed in triplicate are expressed. $^{*}P < 0.05$ as compared with basal level. (**D**) SCC4 cells were pretreated with polymyxin B (poly B, 1 μM) for 30 min followed by stimulation with lipopolysaccharide (LPS) (1 μM) or SDF-1α (100 ng/ml) for 24 h. Media were collected to measure IL-6. Results of four independent experiments performed in triplicate are expressed. $^{*}P < 0.05$ as compared with basal level. $^{#}P < 0.05$ as compared with LPS- or SDF-1α-treated group. (**E**) SCC4 cells were incubated with SDF-1α (100 ng/ml) for indicated time intervals, and cell lysates were then collected, and the mRNA or protein level of CXCR4 was determined by using RT–PCR or western blot analysis, respectively. (**F**) SCC4 cells were incubated with SDF-1α (100 ng/ml) for indicated time intervals, and the cell surface expression of CXCR4 was determined by using flow cytometry. (**G**) SCC4 cells were transfected with human full-length CXCR4 or vector for 48 h; the protein level of CXCR4 was determined using western blot analysis. (**H**) SCC4 cells were transfected with human full-length CXCR4 or vector for 48 h and then incubated with SDF-1α for 24 h. Media were collected to measure IL-6. Results of four independent experiments performed in triplicate are expressed.

SCC4 and SCC9). Treatment of SCC cells with SDF-1α (100 ng/ml) for 24 h induced IL-6 production in a concentration-dependent manner (Figure 1B), and this induction occurred in a time-dependent manner (Figure 1C). After SDF-1α (100 μg/ml) treatment for 24 h, the amount of IL-6 released had increased in oral cancer cells (Figure 1C). To further confirm this stimulation-specific mediation by SDF-1α without lipopolysaccharide contamination, polymyxin B, a lipopolysaccharide inhibitor, was used. We found that polymyxin B (1 μM) completely inhibited lipopolysaccharide (1 μM)-induced IL-6 release.

However, it had no effect on SDF-1α (100 ng/ml)-induced IL-6 release in SCC4 cells (Figure 1D).

SDF-1α–CXCR4 interaction directs the expression of IL-6 in SCC cells

Interaction of SDF-1 with its specific receptor CXCR4 on the surface of prostate cancer cells has been reported to induce the release of cytokine (34). We examined whether SDF-1–CXCR4 interaction was involved in the signal transduction pathway leading to IL-6

expression caused by SDF-1α. Human SCC cells were treated with SDF-1α for different time intervals, and cell lysates were collected. The results from reverse transcriptase–PCR, western blot and flow cytometry indicated that SDF-1α significantly increased mRNA and protein levels and cell surface expression of CXCR4 time dependently (Figure 1E and F). Transfection of SCC4 cells with human full-length CXCR4 increased the CXCR4 expression by western blot analysis (Figure 1G). In addition, overexpression of human full-length CXCR4 increased the SDF-1α-induced IL-6 expression in human SCC cells (Figure 1H). Pretreatment of SCC4 cells for 30 min with CXCR4-specific chemical inhibitor AMD3100 (200, 500 ng/ml), CXCR4-neutralizing antibody (12G5) (10 μg/ml) but not mouse monoclonal immunoglobulin isotype control (isotype antibody) (10 μg/ml) antagonized the SDF-1α-induced IL-6 expression (Figure 2A). Small

interfering RNA (siRNA) against CXCR4 (siCXCR4), but not a mutant form of siCXCR4, specifically inhibited mRNA and protein levels of CXCR4, respectively (Figure 2B). Transient transfection of siCXCR4 but not a mutant form of siCXCR4 effectively inhibited the expression of IL-6 directed by SDF-1α (Figure 2C). On the other hand, the osteoblast-conditioned medium induced the mRNA and protein expression of IL-6 that was reduced by siCXCR4 but not a mutant form of siCXCR4 (Figure 2D). It is well established that osteoblasts cells can synthesize and secrete SDF-1α, which plays an important role in prostate cancer metastasizing to bone (33). Human osteoblasts cells (MG-63) were transfected with the control or SDF-1 siRNA, after which their osteoblast-conditioned medium was collected. The expression of SDF-1 was suppressed by transfection with SDF-1 siRNA (Figure 2E). Additionally, SDF-1 siRNA could markedly block the
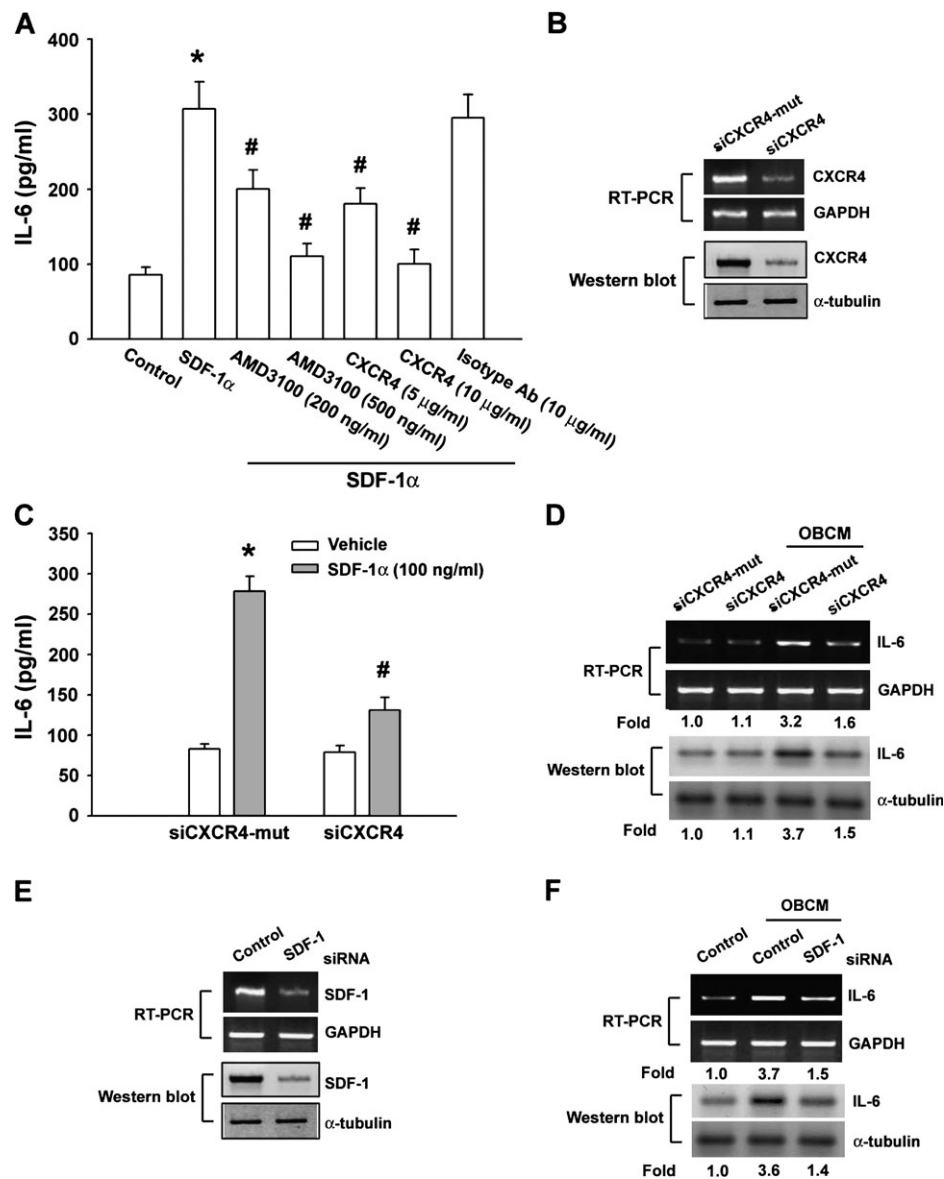
Fig. 2. SDF-1α/CXCR4 mediated the SDF-1α-induced IL-6 expression. (**A**) SCC4 cells were pretreated with AMD3100 (200, 500 ng/ml), CXCR4-neutralizing antibody (12G5; 10 μg/ml) and mouse monoclonal immunoglobulin isotype control (isotype antibody; 10 μg/ml) for 30 min. Media were collected to measure IL-6. (**B**) SCC4 cells were transfected with a mutant form of siCXCR4 or siCXCR4 for 24 h; the mRNA and protein levels of CXCR4 were determined using reverse transcriptase–polymerase chain reaction (RT–PCR) or western blot analysis, respectively. (**C** and **D**) SCC4 cells were transfected with siCXCR4 or a mutant form of siCXCR4 siRNA for 24 h followed by stimulation with SDF-1α (100 ng/ml) or osteoblast-conditioned medium (OBCM; 75%). The medium or mRNA and protein levels of IL-6 were determined by using enzyme-linked immunosorbent assay or RT–PCR and western blot, respectively. (**E**) Osteoblasts (MG-63) were transfected with SDF-1α or control siRNA for 24 h. The mRNA or protein level of SDF-1α determined by using RT–PCR and western blot analysis were suppressed after transfection with SDF-1 siRNA. (**F**) Osteoblasts cells were transfected with SDF-1α or control siRNA for 24 h, the medium was collected as conditioned medium then apply to SCC4 cells and the mRNA or protein level of IL-6 was measured by using RT–PCR or western blot analysis, respectively.

osteoblast-conditioned medium-induced expression of IL-6 in SCC4 cells (Figure 2F). These data suggest that SDF-1 secreted from bone cells plays a key role in the secretion of IL-6 in SCC cells.

ERK signaling pathway is involved in SDF-1α-mediated IL-6 upregulation

As SDF-1α–CXCR4 interaction has been shown to activate several signaling pathways, including phosphatidylinositol 3-kinase/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK), in various cell lines (23,24). We performed western blot analysis to elucidate the signal transduction mechanisms involved in the SDF-1α-induced upregulation of IL-6. SDF-1α activated the ERK1/2 pathway in SCC4 cells, as evidenced by the increase in phosphorylated p42 and p44 (p-ERK) (Figure 3A). Other signaling pathways including p38 MAPK, JNK and Akt were not activated up to 2 h after treatment (Figure 3A). SDF-1α-induced IL-6 production was greatly reduced by treatment with the ERK inhibitor PD98059 (10 μM), but was not affected by SB203580 (a p38 MAPK inhibitor; 10 μM), SP600125 (a JNK inhibitor; 10 μM) or Akt inhibitor (10 μM) (Figure 3B). In addition, transfection of cells with ERK2 but not p38, JNK or Akt mutants also antagonized the potentiating effects of SDF-1α (Figure 3C). Taken together, these data suggest that the activation of the ERK pathway is required for the SDF-1α-induced increase of IL-6 in human SCC cells.

Involvement of NF-κB in SDF-1α-induced IL-6 production

There are NF-κB- and AP-1-binding sites on this IL-6 promoter region (32,35). The increase of IL-6 production by SDF-1α was antagonized by *cis* element decoy agonist NF-κB-binding site (decoy NF-κB ODN) but not by AP-1-binding site (decoy AP-1 ODN) or scrambled decoy (ODN) (Figure 4A). NF-κB activation has been

reported to be necessary for IL-6 induction in macrophages (32). To examine whether NF-κB activation is involved in the signal transduction pathway leading to IL-6 expression caused by SDF-1α, the NF-κB inhibitor PDTC was used. Figure 4B shows that PDTC (30 μM) inhibited the enhancement of IL-6 production induced by SDF-1α. Furthermore, pretreatment of SCC cells with an IκB protease inhibitor [L-1-tosylamido-2-phenylenylethyl chloromethyl ketone (3 μM)] and NF-κB inhibitor peptide (10 μg/ml) also antagonized the potentiating action of IL-6 (Figure 4B). It has been reported that the NF-κB binding site between −72 and −63 was important for the activation of the IL-6 gene (32). NF-κB activation was further evaluated by analyzing the translocation of NF-κB from cytosol to the nucleus, as well as by chromatin immunoprecipitation assay. Treatment of cells with SDF-1α resulted in a marked translocation of p65 and p50 NF-κB from the cytosol to the nucleus (Figure 4C). The *in vivo* recruitment of p65 and p50 to the IL-6 promoter (−288 to −39) was assessed by chromatin immunoprecipitation assay. *In vivo* binding of p65 and p50 to the NF-κB element of the IL-6 promoter occurred as early as 15 min and was sustained to 120 min after SDF-1α stimulation (Figure 4D). The binding of p65 and p50 to NF-κB element by SDF-1α stimulation was attenuated by AMD3100 and PD98059 but not SB203580, SP600125 and Akt inhibitor (Figure 4E). To further confirm the NF-κB element involved in the action of SDF-1α-induced IL-6 expression, transient transfection was performed using the κB promoter–luciferase constructs. SCC4 cells incubated with SDF-1α (100 ng/ml) led to a 3.3-fold increase in κB promoter activity. The increase of κB activity by SDF-1α was antagonized by AMD3100 and PD98059 but not SB203580, SP600125 and Akt inhibitor (Figure 4F). These results suggest that NF-κB activation is necessary for SDF-1α-induced IL-6 production in human SCC cells.
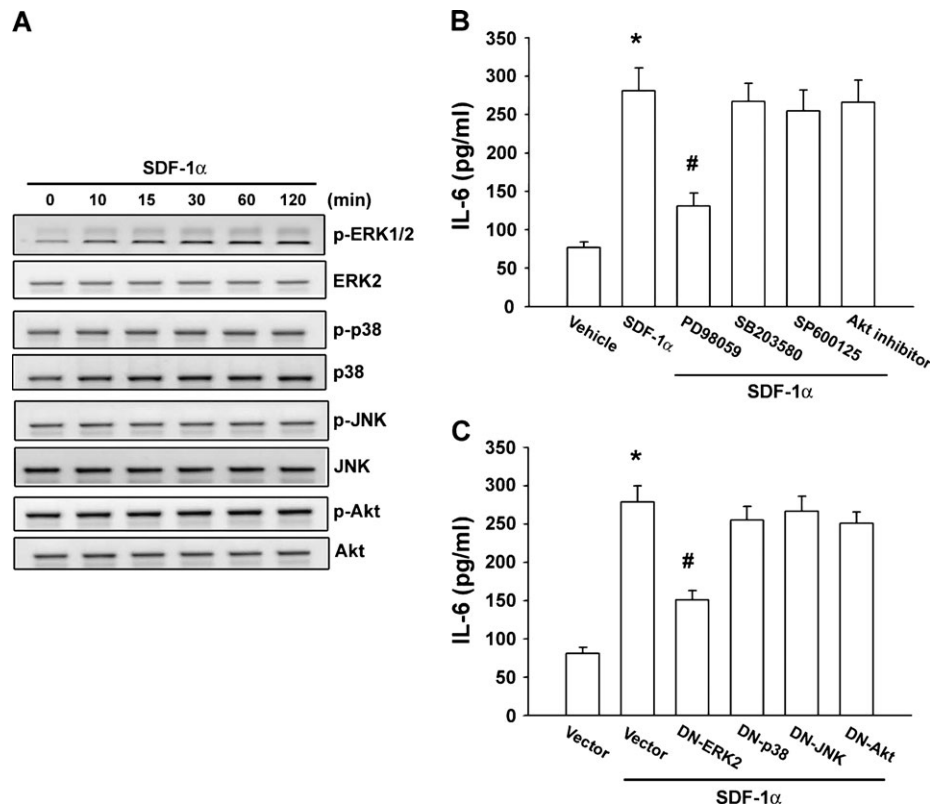
Fig. 3. ERK is involved in the potentiation of IL-6 expression by SDF-1α. SCC4 cells were incubated with SDF-1α (100 ng/ml) for indicated time intervals, and p-ERK, p-p38, p-JNK or p-Akt expression was determined by western blot analysis (**A**). Cell were pretreated for 30 min with PD98059 (10 μM), SB203580 (10 μM), SP600125 (10 μM) and Akt inhibitor (10 μM) (**B**) or transfected with dominant-negative (DN) mutant of ERK, p38, JNK and Akt (**C**) for 24 h followed by stimulation with SDF-1α (100 ng/ml) for 24 h. Media were collected to measure IL-6. Results are expressed as the mean ± SE. *P < 0.05 as compared with vehicle. #P < 0.05 as compared with SDF-1α-treated group.
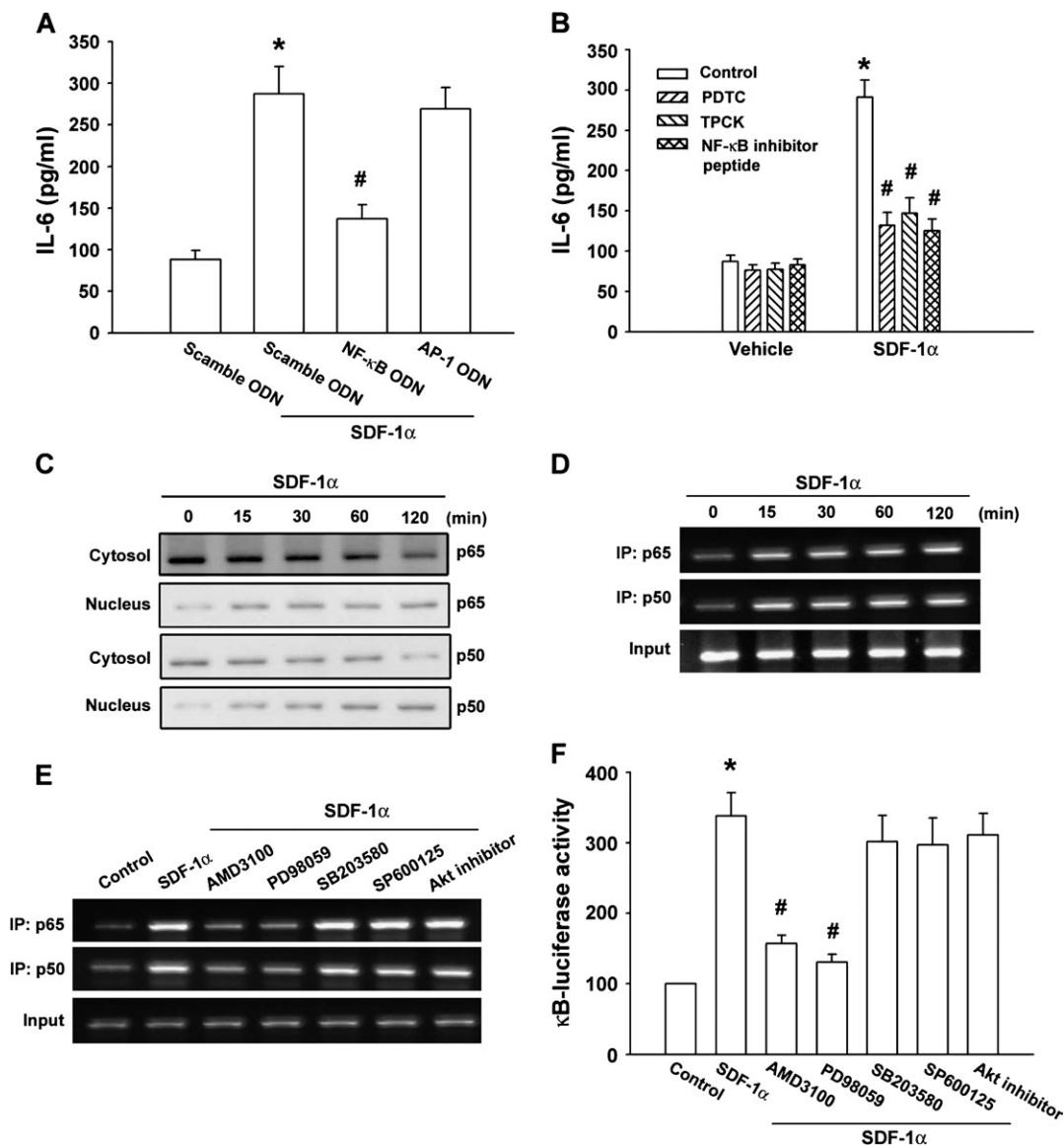
Fig. 4. NF-κB is involved in the potentiation of IL-6 production by SDF-1α. (**A**) SCC4 cells were transfected with NF-κB ODN, AP-1 ODN or scramble ODN before incubation with SDF-1α (100 ng/ml) for 24 h. Media were collected to measure IL-6. (**B**) Cells were pretreated for 30 min with PDTC (60 μM), L-1-tosylamido-2-phenylenylethyl chloromethyl ketone (3 μM) and NF-κB inhibitor peptide (10 μg/ml) followed by stimulation with SDF-1α (100 ng/ml) for 24 h. Media were collected to measure IL-6. Results of four independent experiments performed in triplicate are expressed. *$P < 0.05$ as compared with control. #$P < 0.05$ as compared with SDF-1α-treated group. (**C**) Cells were treated with SDF-1α (100 ng/ml) for indicated time intervals, and the levels of cytosolic and nuclear p65 or p50 were determined by immunoblotting with p65 or p50 specific antibodies, respectively. (**D** and **E**) Cells were treated with SDF-1α (100 ng/ml) for the indicated time intervals or pretreated with AMD3100 (500 ng/ml), PD98059 (10 μM), SB203580 (10 μM), SP600125 (10 μM) and Akt inhibitor (10 μM) and then stimulated with SDF-1α (100 ng/ml) for 60 min, and chromatin immunoprecipitation assay was then performed. Chromatin was immunoprecipitated with anti-p65 or anti-p50 antibody. One percent of the precipitated chromatin was assayed to verify equal loading (input). (**F**) Cells were transfected with κB-luciferase expression vector and then pretreated with AMD3100 (500 ng/ml), PD98059 (10 μM), SB203580 (10 μM), SP600125 (10 μM) and Akt inhibitor (10 μM) for 30 min before incubation with SDF-1α (100 ng/ml) for 24 h. Luciferase activity was then assayed. Results are representative of at least three independent experiments. *$P < 0.05$ as compared with control. #$P < 0.05$ as compared with SDF-1α-treated group.

SDF-1α causes an increase in IKKα/β phosphorylation and IκBα phosphorylation

We further examined the upstream molecules involved in SDF-1α-induced NF-κB activation. Stimulation of cells with SDF-1α induced IKKα/β phosphorylation in a time-dependent manner (Figure 5A). Treatment of SCC cells with SDF-1α also caused IκBα phosphorylation in a time-dependent manner (Figure 5B). Next, we further examined p65 phosphorylation at Ser[276] by SDF-1α in SCC cells. Treatment of cells with SDF-1α induced p65 phosphorylation at Ser[276] in a time-dependent manner (Figure 5C). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited SDF-1α-induced IL-6 production (Figure 5D). Pretreatment of cells with AMD3100 or PD98059 attenuated SDF-1α-induced IκBα, IKKα/β and p65 phosphorylation (Figure 5E). These data suggest that IKKα/β and p65 activation is involved in SDF-1α-induced IL-6 production in human SCC cells.

Conditioned medium from SCC4 supports osteoclast differentiation

Forty-eight hour-conditioned medium (containing serum) from SCC4 cells diluted 50% in Dulbecco's modified Eagle's medium was added to cultures of human PBMCs. SCC4-conditioned medium stimulated osteoclast formation by using TRAP staining (Figure 6A, upper
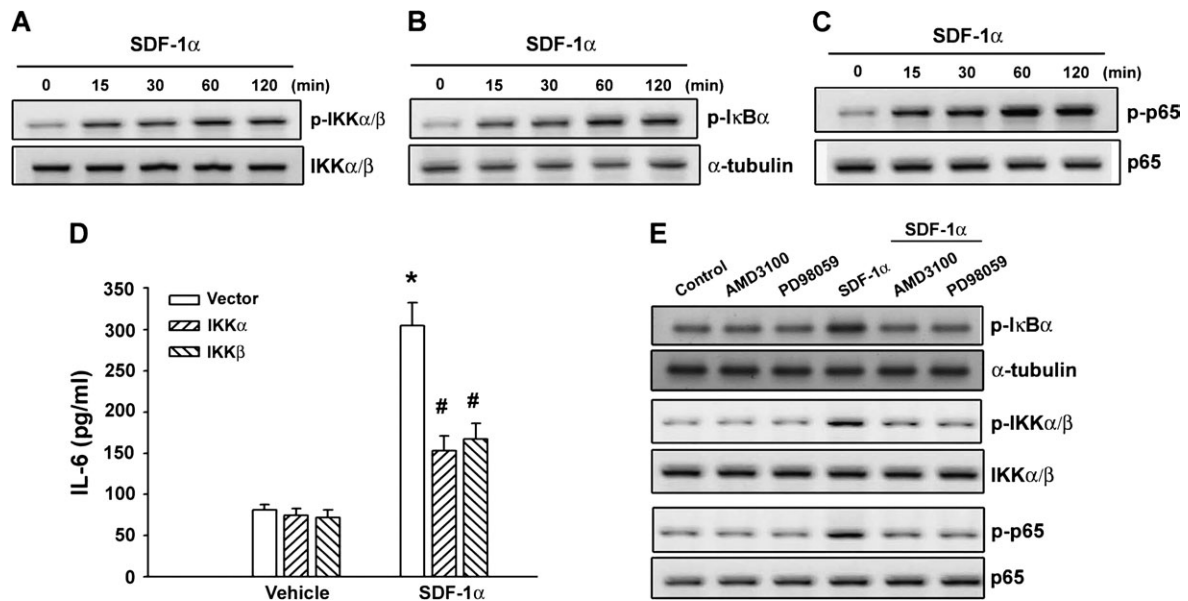
Fig. 5. SDF-1α induces IKKα/β activation, IκBα phosphorylation and p65 Ser^{276} phosphorylation in SCC cells. SCC4 cells were incubated with SDF-1α (100 ng/ml) for indicated time intervals; cell lysates were then immunoblotted with an antibody specific for phosphor-IKKα/β (**A**), phosphor-IκBα (**B**) and p65 phosphorylated at Ser^{276} (**C**) antibodies, respectively. Cells were transfected with IKKα, IKKβ mutant or vector for 24 h followed by stimulation with SDF-1α for 24 h. Media were collected to measure IL-6 (**D**). Results are representative of at least three independent experiments. $^*P < 0.05$ as compared with control. $^#P < 0.05$ as compared with SDF-1α-treated group. Cells were pretreated with AMD3100 (500 ng/ml) or PD98059 (10 μM) and then stimulated with SDF-1α (100 ng/ml) for 60 min, and phosphor-IKKα/β, phosphor-IκBα and p65 phosphorylated at Ser^{276} were then determined by western blot (**E**).

panel). In addition, the TRAP-positive multinucleated cells expressed the resorbing activity (Figure 6A, lower panel). On the other hand, the control medium did not increase the osteoclastogenesis (A549-conditioned medium was used for positive control) (34) (Figure 6B). These data suggest that SCC4 cells secrete factors that stimulate osteoclast formation. We next investigated whether the effects on osteoclasts of SCC4-conditioned medium were due to IL-6 production by tumor cells using commercially available IL-6-neutralizing antibody. The antibodies significantly reduced the number of osteoclasts induced by conditioned medium from SCC4 (Figure 6C). However, IL-6 antibody treatment did not fully suppress osteoclast formation to control levels (Figure 6C), suggesting that SCC4 supports osteoclast formation by IL-6-dependent and IL-6-independent mechanisms. We next examined the IL-6-independent component of the pro-osteoclastogenic activity secreted by SCC4 cells. RANKL, a central regulator of osteoclast formation and activity (36), was an obvious candidate factor for the IL-6-independent component. RANK-Fc (200 ng/ml) was added to SCC4-conditioned medium blocking SCC4-conditioned medium-induced osteoclast formation (Figure 6D). The IL-6 antibody plus RANKL-Fc completely blocked the SCC4-conditioned medium-induced osteoclast formation. These data suggest that secreted RANKL is responsible for the IL-6-independent effects on osteoclast formation. On the other hand, both transforming growth factor-β1 and IL-1 antibodies had no effect on SCC4-conditioned medium-induced osteoclast formation.

Discussion

Oral carcinomas, especially oral SCC, frequently invade into maxillary or mandibular bone, and bone invasion is a more common clinical problem in patients with oral carcinomas. However, its mechanism is poorly understood. Recent studies have revealed that bone resorption by osteoclasts is an important step in the process of bone invasion and metastasis in several malignancies including oral carcinomas (1–3). Although various cytokines, i.e. IL-1, IL-6, IL-11, transforming growth factor, tumor necrosis factor and parathyroid hormone-related protein have been shown to activate osteoclastic bone resorption (31,37), it remains unclear whether these cytokines that can be produced by tumor cells may be directly involved in bone invasion and

metastasis of malignancies. Only parathyroid hormone-related protein has been reported to play an important role in the formation of bone metastasis of human lung and breast carcinoma cells (2).

IL-6 is expressed by a number of cancer cell lines *in vitro*. A correlation is observed between tumor cell expression of IL-6 and metastatic potential (38). Here, we found that human SCC (HSC3, SCC4 and SCC9) expressed IL-6 mRNA and protein. We hypothesized that SDF-1 and its CXCR4 receptor would help to direct the bone-specific invasion of oral SCC cells. In this study, we found that osteoblast-derived SDF-1 induced expression of IL-6 in human SCC cells. Overexpression of human full-length CXCR4 increased SDF-1-induced IL-6 production. We also used CXCR4-specific chemical inhibitor AMD3100 and CXCR4-neutralizing antibody to determine the role of CXCR4 and found that it inhibited SDF-1α-induced IL-6 expression, indicating the possible involvement of CXCR4 in SDF-1α-induced IL-6 expression in SCC cells. This was further confirmed by the result that the siCXCR4 inhibited the enhancement of IL-6 production by SDF-1α, indicating the involvement of SDF-1–CXCR4 interaction in SDF-1α-mediated induction of IL-6.

A variety of growth factors stimulate the expression of IL-6 genes via signal transduction pathways that converge to activate NF-κB complex of transcription factors. MAPK pathways ERK1/2, JNK and p38 induce the expression of NF-κB transcription factors (39). We found that SDF-1α enhanced ERK1/2 phosphorylation without obvious changes of the phosphorylation of Akt and other MAPK pathways (e.g. JNK and p38) in human SCC cells. Previous studies have revealed that SDF-1α treatment activates ERK1/2 in human lung cancer cells, astrocytes and glioblastoma cells (19,21,40). The SDF-1α-directed IL-6 production was effectively inhibited by PD98059, but not by SB203580, SP600125 or the Akt inhibitor. This was further confirmed by the results that the dominant-negative mutant of ERK, but not p38, JNK and Akt, inhibited the enhancement of IL-6 production by SDF-1α. Recently, hepatitis B viral HBx was shown to induce matrix metalloproteinase-9 (MMP-9) gene expression through the activation of the ERK and Akt pathways (41). Liang *et al.* (42) suggested that the ERK pathway was required for IL-1-induced MMP-9 expression. Our data indicate that ERK might play an important role in the expression of IL-6 of human SCC cells.
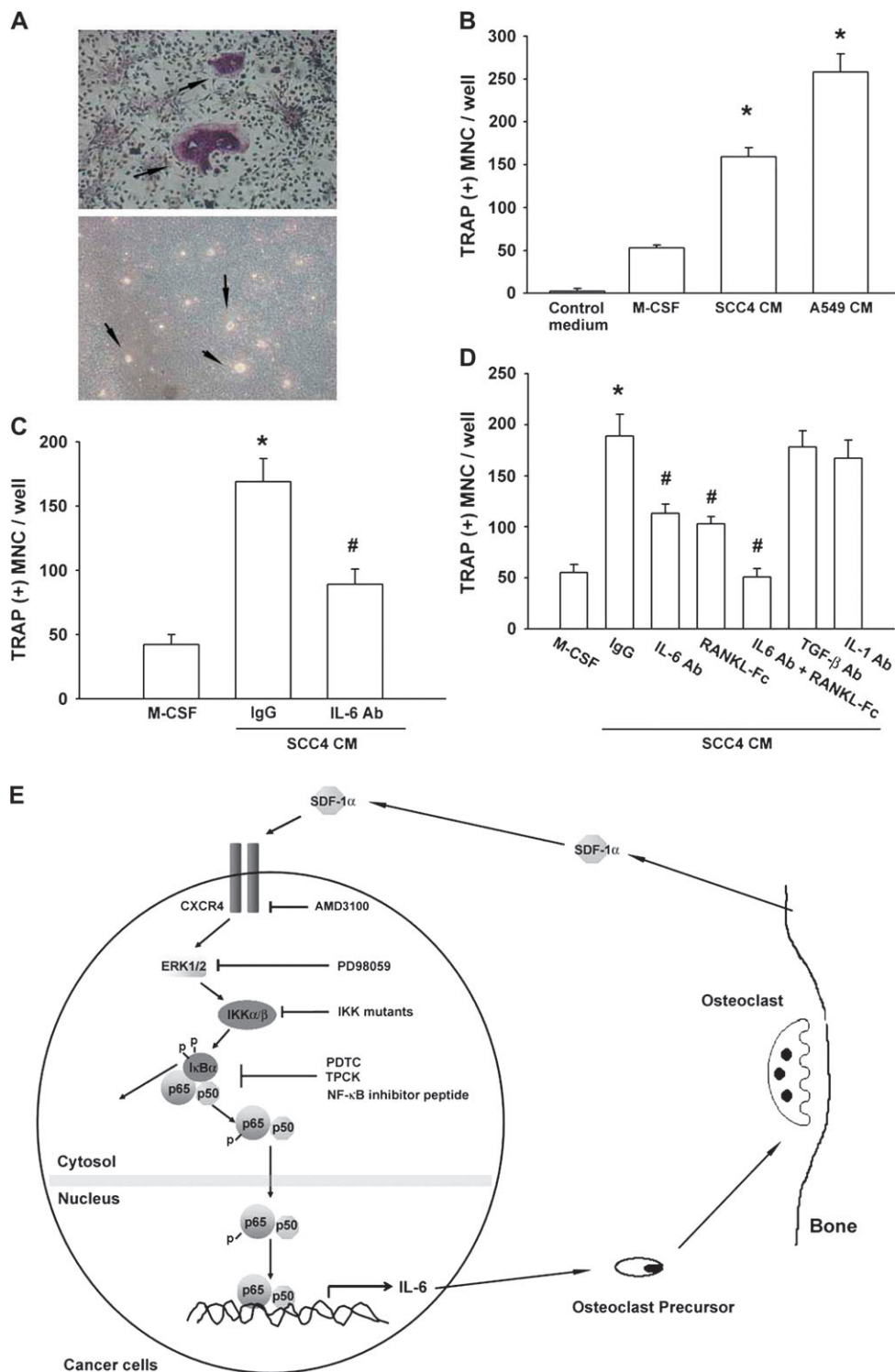
Fig. 6. Conditioned medium from SCC4 cells supports osteoclast differentiation. Conditioned medium from SCC4 cells was added to culture of PBMC. Half the medium was replaced thrice per week and cultures were terminated on day 10. The plates were stained for TRAP (**A**, upper panel). On the other hand, the PBMCs were cultured on osteoclast activity assay substrate plate and the cells were removed after 10 days (A, lower panel). The arrow heads show the osteoclast resorbing pit. (**B**) Control medium, control medium plus macrophage colony-stimulating factor, SCC4-conditioned medium or A549-conditioned medium was added to culture of PBMC. Half the medium was replaced thrice per week and cultures were terminated on day 10. The plates were stained for TRAP. Note that SCC4- and A549-conditioned medium but not control medium stimulated TRAP-positive multinucleated cell. (**C**) Anti-IL-6 antibody (200 μg/ml) but not isotype IgG inhibited the SCC4-conditioned medium-induced osteoclastogenesis. (**D**) IL-6 antibody (200 μg/ml), RANKL-Fc (200 ng/ml), IL-6 antibody plus RANKL-Fc, transforming growth factor-β antibody (200 μg/ml), IL-1 antibody (200 μg/ml) were added to SCC4-conditioned medium; IL-6 antibody, RANKL-Fc, IL-6 antibody plus RANKL-Fc but not TGF-β or IL-1 antibody blocked the SCC4-conditioned medium-induced osteoclastogenesis. $^*P < 0.05$ as compared with control. $^\#P < 0.05$ as compared with IgG-treated group. (**E**) Schematic representation of the signaling pathways involved in the osteoblast-derived SDF-1α-induced IL-6 production and promotion of osteoclastogensis in human SCC cells. Osteoblasts release SDF-1α and then activate CXCR4, ERK and IKKα/β, leading to the activation of NF-κB on the IL-6 promoter, initiation of IL-6 mRNA and protein release and promotion of osteoclastogenesis.

There are several binding sites for a number of transcription factors including NF-κB, cAMP response element-binding protein, NF-IL-6 and AP-1 box in the 5′ region of the IL-6 gene (32,35). To date, two transcription factors (NF-κB and the AP-1) appear to be responsive to the SDF-1 (43). In this study, NF-κB but not AP-1 modulated SDF-1α-induced IL-6 activity in SCC cells. The results of this study also show that NF-κB activation contributes to SDF-1α-induced IL-6 production in SCC cells and that the inhibitors of the NF-κB-dependent signaling pathway, including PDTC, L-1-tosylamido-2-phenylenylethyl chloromethyl ketone or NF-κB inhibitor peptide, inhibited SDF-1α-induced IL-6 expression. In an inactivated state, NF-κB is normally held in the cytoplasm by the inhibitor protein IκB. Upon stimulation, such as by tumor necrosis factor-α, IκB proteins become phosphorylated by the multisubunit IKK complex, which subsequently targets IκB for ubiquitination, and then are degraded by the 26S proteasome. Finally, the free NF-κB translocates to the nucleus, where it activates the responsive gene. In the present study, we found that treatment of SCC4 cells with SDF-1α resulted in increases in IKKα/β phosphorylation and activity, p65 and p50 translocation from the cytosol to the nucleus and the binding of p65 and p50 to the NF-κB element on the IL-6 promoter. Using transient transfection with κB-luciferase as an indicator of NF-κB activity, we also found that SDF-1α induced an increase in NF-κB activity. The IKKs can be stimulated by various proinflammatory stimuli, including IL-1β, peptidoglycan and thrombin (44). These extracellular signals activate the IKK complex, which is comprised of catalytic subunits (IKKα and IKKβ) and a linker subunit (IKKγ/NEMO). This kinase complex in turn phosphrylates IκBα at Ser^{32} and Ser^{36} and signals for ubiquitin-related degradation. The released NF-κB is then translocated into the nucleus where it promotes NF-κB-dependent transcription. The findings of our experiments show that pretreatment of SCC4 cells with AMD3100 or PD98059 antagonized the increase of IκBα, IKKα/β and p65 phosphorylation by SDF-1α. Based on these findings, we suggest that the CXCR4/ERK pathway is involved in SDF-1α-induced IκBα, IKKα/β and p65 activation. Similar findings have reported that the activation of p-ERK precedes and is required for activation of IKKα/β, p65 and NF-κB (45,46). Our previous data showed that SDF-1α enhanced motility and upregulated MMP-9 and β3 integrin via ERK/IKKαβ and NF-κB pathway in human lung cancer cells (45,46). Here, we also found that ERK or NFκB inhibitors and IKKα or IKKβ mutants also inhibited the SDF-1α-induced motility and upregulated MMP-9 and β3 integrin in SCC4 cells (data not shown). Therefore, the same signaling pathway mediates the motility and the expression of MMP-9 and integrins in SCC cells.

In conclusion, we present here a novel mechanism of SDF-1α/CXCR4-directed invasion of oral SCC cells by upregulation of IL-6 production and promotion of osteoclastogenesis. The identification of SDF-1α from bone as a potential stimulatory factor of IL-6 during human cancer cell invasion to bone and promotion of osteoclastogenesis may help understand the mechanisms involved in the aggressive potential of human SCC cells (Figure 6E). In addition, the identification of SDF-1α–CXCR4 interaction as an important factor in the invasiveness of human SCC cells may implicate potential therapeutic approaches for bone invasion from oral SCC cells.

Funding

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