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Journal of Cellular Biochemistry 112:933–941 (2011)

Journal of Cellular Biochemistry

RANKL Increases Migration of Human Lung Cancer Cells Through Intercellular Adhesion Molecule-1 Up-Regulation

Li-Mien Chen,[1,2] Chia-Hua Kuo,[3] Tung-Yuan Lai,[4,5] Yueh-Min Lin,[6] Cheng-Chuan Su,[7,8] His-Hsien Hsu,[9] Fuu-Jen Tsai,[5] Chang-Hai Tsai,[10] Chih-Yang Huang,[5,11,12*] and Chih-Hsin Tang[11,13*]

[1] *Center of General Education, Central Taiwan University of Science & Technology, Taichung, Taiwan*
[2] *Department of Internal Medicine, Armed Force Taichung General Hospital, Taichung, Taiwan*
[3] *Laboratory of Exercise Biochemistry, Taipei Physical Education College, Taipei, Taiwan*
[4] *College of Chinese Medicine, School of Post-Baccalaureate Chinese Medicine, China Medical University, Taichung, Taiwan*
[5] *Department of Chinese Medicine, China Medical University Hospital, Taichung, Taiwan*
[6] *Department of Pathology, Changhua Christian Hospital, Changhua, Taiwan*
[7] *Departments of Clinical Pathology and Anatomic Pathology, Buddhist Dalin Tzu Chi General Hospital, Chiayi, Taiwan*
[8] *Department of Pathology, School of Medicine, Tzu Chi University, Hualien, Taiwan*
[9] *Division of Colorectal Surgery, Mackay Memorial Hospital, Taipei, Taiwan*
[10] *Department of Healthcare Administration, Asia University, Taichung, Taiwan*
[11] *Graduate Institute of Basic Medical Science, China Medical University and Hospital, Taichung, Taiwan*
[12] *Department of Health and Nutrition Biotechnology, Asia University, Taichung, Taiwan*
[13] *Department of Pharmacology, School of Medicine, China Medical University and Hospital, Taichung, Taiwan*

ABSTRACT

Invasion of distant tissues by tumor cells is the primary cause of therapeutic failure in the treatment of malignant lung cancer cells. Receptor activator of nuclear factor-κB ligand (RANKL) and its receptor, RANK, play a key role in osteoclastogenesis and tumor metastasis. Intercellular adhesion molecule-1 (ICAM-1, also called CD54), a member of the immunoglobulin supergene family, is an inducible surface glycoprotein that mediates adhesion-dependent cell-to-cell interactions. The effects of RANKL on cell migration and ICAM-1 expression in human lung cancer cells are largely unknown. We found that RANKL directed the migration and increased ICAM-1 expression in human lung cancer (A549) cells. Pretreatment of A549 cells with the MAPK kinase (MEK) inhibitor PD98059 or U0126 inhibited RANKL-mediated migration and ICAM-1 expression. Stimulation of cells with RANKL increased the phosphorylation of MEK and extracellular signal-regulating kinase (ERK). In addition, an NF-κB inhibitor (PDTC) and IκB protease inhibitor (TPCK) also inhibited RANKL-mediated cell migration and ICAM-1 up-regulation. Taken together, these results suggest that the RANKL and RANK interaction acts through MEK/ERK, which in turn activates NF-κB, resulting in the activation of ICAM-1 and contributing to the migration of human lung cancer cells. J. Cell. Biochem. 112: 933–941, 2011.

KEY WORDS: RANKL; RANK; MIGRATION; LUNG CANCER; ERK

All authors have no financial or personal relationships with other people or organizations that could inappropriately influence our work.

Grant sponsor: Taiwan Department of Health, China Medical University Hospital Cancer Research of Excellence; Grant number: DOH99-TD-C-111-005; Grant sponsor: Taiwan Department of Health Clinical Trial and Research Center of Excellence; Grant number: DOH99-TD-B-111-004.

*Correspondence to: Chih-Hsin Tang and Chih-Yang Huang, Department of Pharmacology, College of Medicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung, Taiwan. E-mail: chtang@mail.cmu.edu.tw, cyhuang@mail.cmu.edu.tw

Received 19 September 2010; Accepted 20 December 2010 • DOI 10.1002/jcb.23009 • © 2010 Wiley-Liss, Inc.
Published online 29 December 2010 in Wiley Online Library (wileyonlinelibrary.com).

Lung cancer is the leading cause of cancer-related mortality in both men and women [Jemal et al., 2005], with 1.2 million new cases diagnosed every year and 1 million deaths being recorded worldwide [Gridelli et al., 2003]. Non-small cell lung cancer (NSCLC) affects approximately 80% of all lung cancer patients. Most patients present with locally advanced (37%) or metastatic (38%) disease at the time of diagnosis [Jemal et al., 2005], and a large percentage of those diagnosed with early-stage disease eventually experience recurrence of metastatic disease. Thus, the high degree of invasiveness of NSCLC to regional lymph nodes, liver, adrenal glands, contralateral lung, brain, and bone marrow may play a key role in its biological virulence [Jemal et al., 2005].

Receptor activator of nuclear factor-κB (RANK) ligand (RANKL) is a tumor necrosis factor-ligand superfamily member that is essential for osteoclast formation, function, and survival [Boyle et al., 2003]. RANKL interacts with the cell surface receptor RANK and in turn recruits tumor necrosis factor receptor-associated factors (TRAF) 1, 2, 3, 5, and 6 [Darnay et al., 1998]. Receptor deletion analysis has shown that sequential recruitment of TRAF6 and NF-κB-inducing kinase by RANK leads to NF-κB activation, and recruitment of TRAF2 leads to c-Jun N-terminal kinase (JNK) activation [Lee et al., 1997; Darnay et al., 1999]. RANKL also activates NF-κB, JNK, and p38 and p44/p42 mitogen-activated protein kinase (MAPK) [Lee et al., 1997; Darnay et al., 1999]. However, it is still not fully understood how this cytokine is involved in tumor metastasis.

Tumor invasion and metastasis are critical in determining the aggressive phenotype of human cancers. Mortality in cancer patients principally results from the metastatic spread of cancer cells to distant organs [Gupta and Massague, 2006]. To facilitate cell motility, invading cells need to change the cell–cell adhesion properties, rearrange the extracellular matrix environment, suppress anoikis, and reorganize their cytoskeletons [Desgrosellier and Cheresh, 2010]. Cell adhesion molecules belonging to the integrin, cadherin, and immunoglobulin superfamilies have been implicated in tumor progression [Makrilia et al., 2009]. Intercellular adhesion molecule-1 (ICAM-1, also called CD54), a member of the immunoglobulin supergene family, is an inducible surface glycoprotein that mediates adhesion-dependent cell-to-cell interactions [Zimmerman and Blanco, 2008; Lawson and Wolf, 2009]. The extracellular domain of ICAM-1 is essential for the transendothelial migration of leukocytes from capillary beds into tissues [Duperray et al., 1997], and ICAM-1 may also facilitate movement (or retention) of cells through the extracellular matrix [Duperray et al., 1997]. ICAM-1 plays an important role in lung cancer cell invasion [Huang et al., 2004]. ICAM-1 antibody or antisense ICAM-1 cDNA also reduces the invasiveness of breast cancer cells [Rosette et al., 2005]. Therefore, ICAM-1 might play a critical role in tumorigenesis, and its disruption may prevent metastasis.

RANKL modulates cell migration and invasion in human cancer cells [Armstrong et al., 2008; Heymann et al., 2008; Virk et al., 2009]. Interaction of RANKL with its specific receptor RANK on the surface of cancer cells induces cancer invasion [Armstrong et al., 2008; Heymann et al., 2008]. The effect of RANKL on ICAM-1 expression and migration activity in human lung cancer cells is, however, mostly unknown. Here we describe a phenomenon whereby the RANKL and RANK interaction increased the migration and expression of ICAM-1 in human lung cancer cells. In addition, we show that MAPK kinase (MEK), ERK, and NF-κB signaling pathways may be involved in the increase in ICAM-1 expression and cell migration by RANKL.

MATERIALS AND METHODS

MATERIALS

Protein A/G beads; horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG; and rabbit polyclonal antibodies specific for p-MEK, MEK, p-ERK, ERK, p65, and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies specific for RANKL, ICAM-1, and osteoprotegerin (OPG) were purchased from R&D Systems (Minneapolis, MN). U0126, PD98059, TPCK, and PDTC were purchased from Calbiochem (San Diego, CA). Rabbit polyclonal antibody specific for phospho-p65 (Ser^{536}) was purchased from Cell Signaling (Danvers, MA). Recombinant human RANKL was purchased from PeproTech (Rocky Hill, NJ). The MEK1 dominant-negative mutant was a gift from Dr. W. M. Fu (National Taiwan University, Taipei, Taiwan). The ERK2 dominant-negative mutant was a gift from Dr. M. Cobb (University of Texas Southwestern Medical Center, Dallas, TX). pSV-β-galactosidase vector and the luciferase assay kit were purchased from Promega (Madison, MA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

CELL CULTURE

The human lung adenocarcinoma cell lines A549, H928, and H1299 were obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium/Nutrient Mixture Ham's F12 (DMEM/F12), which was supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin at 37°C with 5% CO_2.

MIGRATION ASSAY

The migration assay was performed using Transwell inserts (Costar, NY; pore size, 8 μm) in 24-well dishes. Before the migration assay was carried out, cells were pretreated for 30 min with different concentrations of inhibitors, including U0126, PD98059, PDTC, and TPCK, or vehicle control (0.1% DMSO). The concentrations of the inhibitors did not affect A549 cell death as indicated by a cell viability assay (data not shown). Approximately 1×10^4 cells in 200 μl of serum-free medium were placed in the upper chamber, and 300 μl of the same medium containing RANKL was placed in the lower chamber. The plates were incubated for 24 h at 37°C in 5% CO_2, and then cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Each clone was plated in triplicate in each experiment, and each experiment was repeated at least three times. The number of invading cells in each experiment was adjusted by the cell viability assay to correct for proliferation effects of RANKL treatment (corrected invading cell number = counted invading cell number/percentage of viable cells) [Fong et al., 2008].

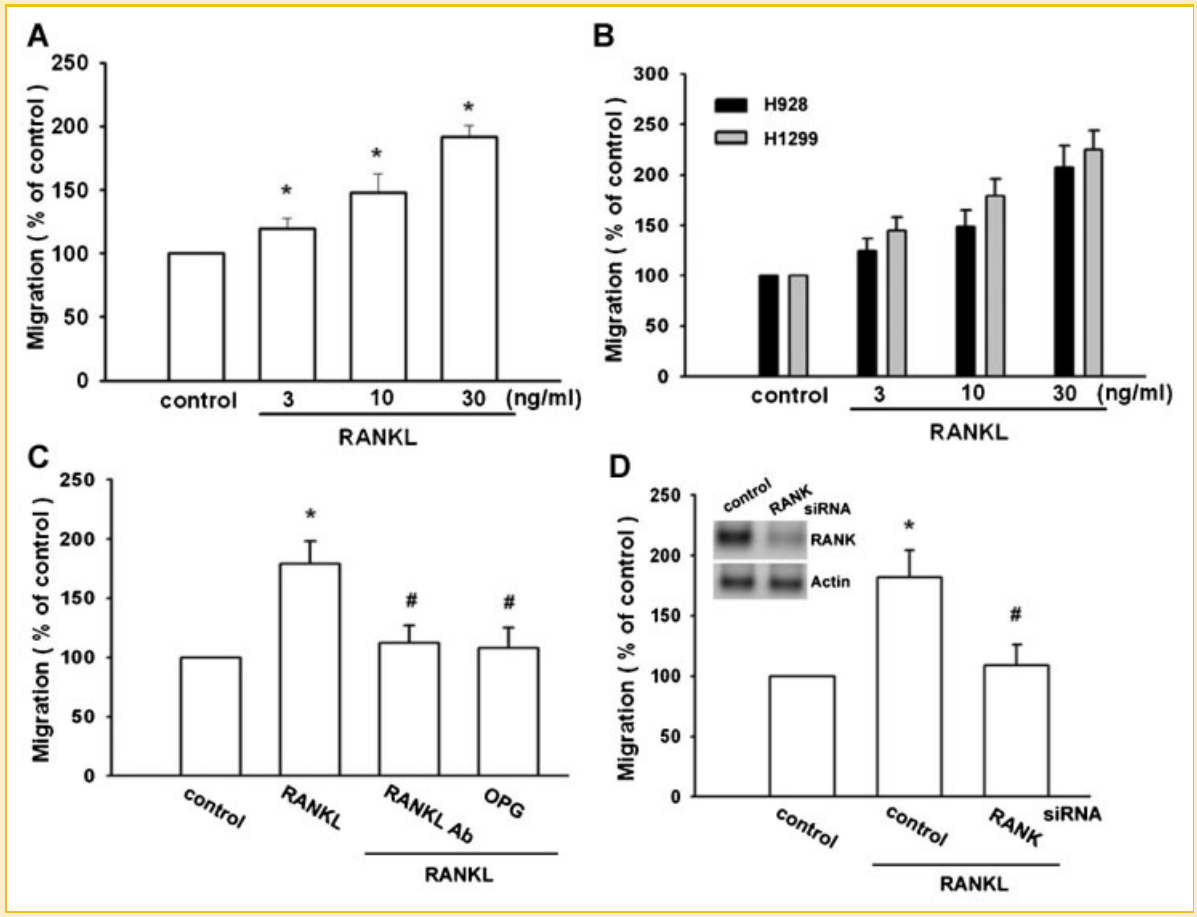

Fig. 1. RANKL/RANK axis-directed migration of human lung cancer cells. A: A549 cells were incubated with various concentrations of RANKL, and in vitro migration activity measured with the Trandswell after 24 h. B: H928 and H1299 were incubated with various concentrations of RANKL, and in vitro migration activity measured with the Trandswell after 24 h. Cells were pretreated for 30 min with RANKL Ab (10 μg/ml) and OPG (30 ng/ml) (C) or transfected with RANK siRNA (D) for 24 h. Then they were followed by stimulation with RANKL, and in vitro migration was measured with the Transwell after 24 h. Results are expressed as the mean ± SE. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with RANKL-treated group.

WESTERN BLOT ANALYSIS

The cellular lysates were prepared as described [Tang et al., 2008]. Proteins were resolved by SDS–PAGE and transferred to Immobilon polyvinyldifluoride membranes. The blots were blocked with 4% BSA for 1 h at room temperature and were then probed with rabbit anti-human antibodies against MEK, ERK, p-MEK, and p-ERK (1:1,000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a horseradish peroxidase–conjugated donkey anti-rabbit secondary antibody (1:1,000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

SMALL-INTERFERING RNA (siRNA) TRANSFECTION

The siRNAs against human RANK, ICAM-1, ERK1 and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with siRNA (100 nM) using Lipofectamine 2000 (LF2000; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions [Tang et al., 2010].

QUANTITATIVE REAL-TIME PCR (qPCR)

qPCR analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA). Total cDNA (100 ng) was added to each 25-μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially; GAPDH was used as an internal control (Applied Biosystems. qPCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

IMMUNOFLUOROCYTOCHEMISTRY

Cells were cultured on 12-mm coverslips. After treatment with RANKL, cells were fixed with 4% paraformaldehyde at room temperature. After 30 min, 4% nonfat milk in PBS containing 0.5%

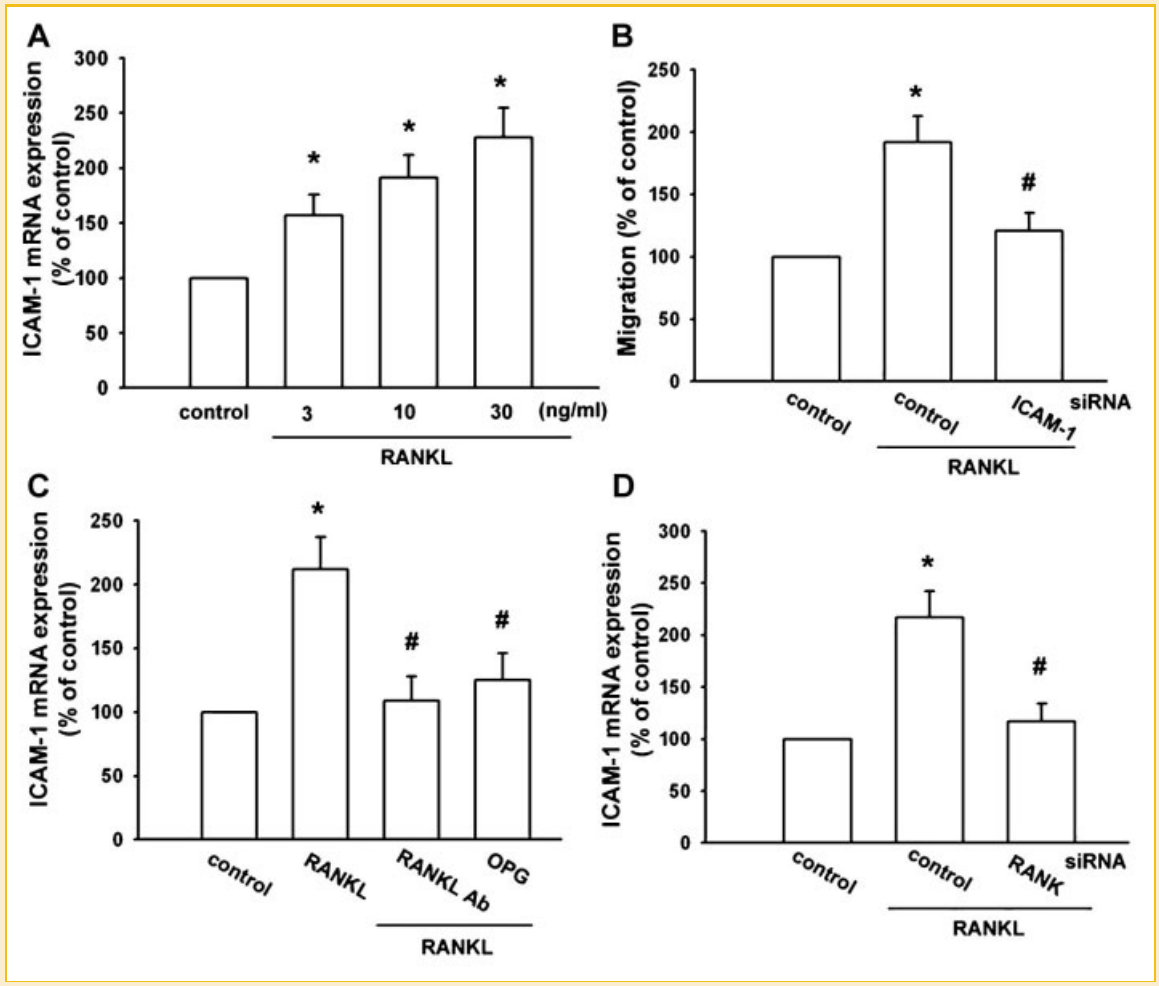

Fig. 2. RANKL-directed migration activity of human lung cancer cells involves up-regulation of ICAM-1. A: A549 cells were incubated with RANKL for 24 h, and the ICAM-1 mRNA expression was examined by qPCR. B: Cells were transfected with ICAM-1 siRNA for 24 h followed by stimulation with RANKL, and in vitro migration measured with the Transwell after 24 h. Cells were pretreated for 30 min with RANKL Ab (10 μg/ml) and OPG (30 ng/ml) (C) or transfected with RANK siRNA (D) for 24 h. Then they were followed by stimulation with RANKL, and ICAM-1 mRNA expression was measured with the qPCR. Results are expressed as the mean ± SE. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with RANKL-treated group.

Triton X-100 was added to the cells. The cells were then incubated with rabbit anti-p65 (1:100) for 1 h and then with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:500; Leinco Technology Inc., St Louis, MO) for 1 h. The FITC was detected using a Zeiss fluorescence microscope.

CHROMATIN IMMUNOPRECIPITATION ASSAY

Chromatin immunoprecipitation analysis was performed as described [Huang and Chen, 2005]. DNA immunoprecipitated by antibodies against p65 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 ultraviolet light. The primers 5′-AGACCTTAGCGCGGTGTAGA-3′ and 5′-AGTAGCAGAGGAGCT-CAGCG-3′ were used to amplify across the ICAM-1 promoter region (−346 to −24) [Huang and Chen, 2005].

REPORTER GENE ASSAY

Human lung cancer cells were co-transfected with 0.8 μg κB-luciferase plasmid and 0.4 μg β-galactosidase expression vector. A549 cells were grown to 80% confluence in 12-well plates and were transfected on the following day with LF2000. DNA and LF2000 were premixed for 20 min and then applied to cells. After transfection for 24 h, the cells were incubated with the indicated agents. After a further 24-h incubation, the medium was removed, and the cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI) was added to each well, and cells were scraped from the dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into the 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 the

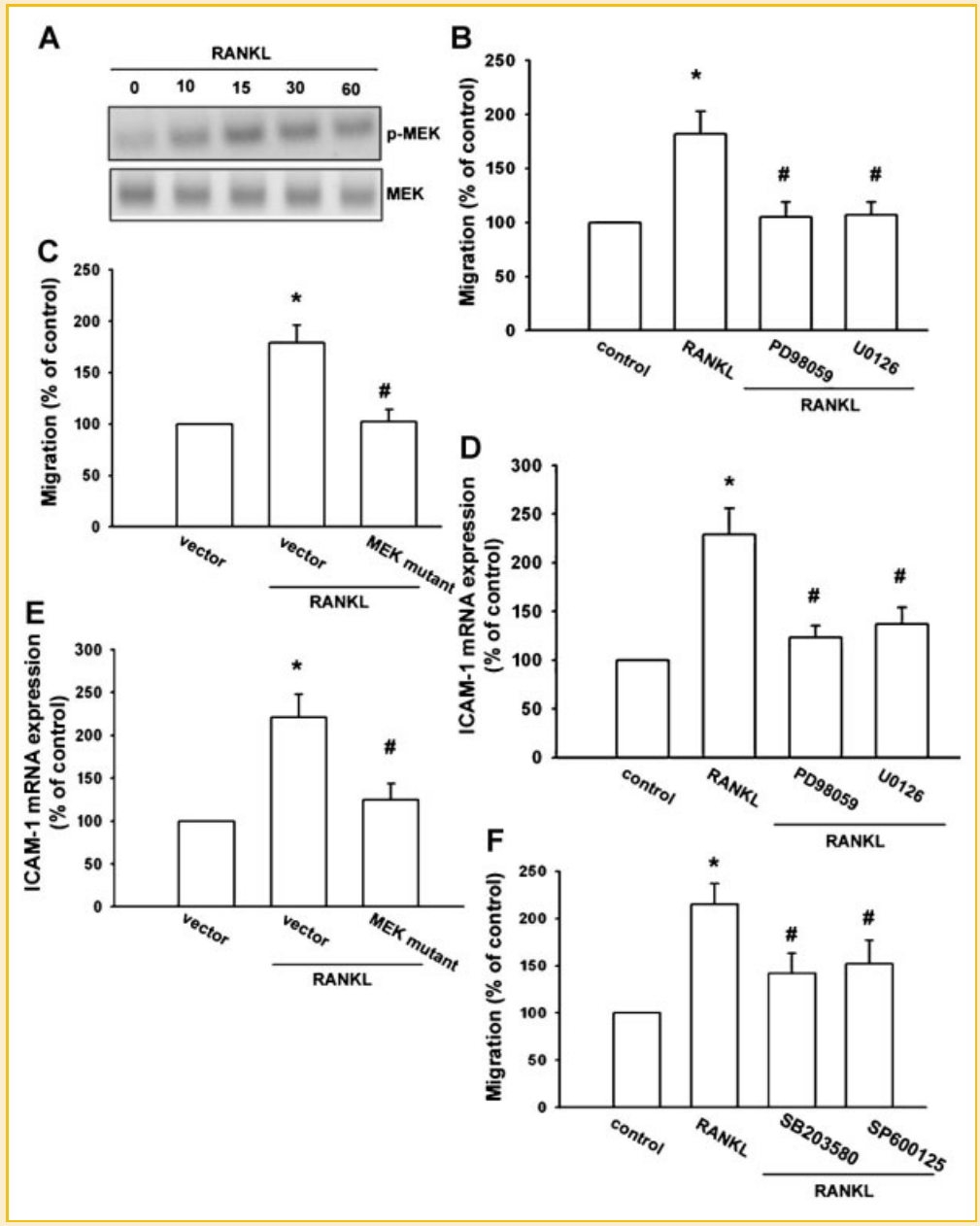

Fig. 3. MEK is involved in RANKL-induced migration and ICAM-1 up-regulation in human lung cancer cells. A: A549 cells were incubated with RANKL for indicated time intervals, and p-MEK was examined by Western blot analysis. B,C: A549 cells were pretreated for 30 min with U0126 and PD98059 or transfected with dominant negative (DN) mutant of MEK1 for 24 h followed by stimulation with RANKL, and in vitro migration was measured with the Transwell after 24 h. D,E: A549 cells were pretreated for 30 min with U0126 (30 μg/ml) and PD98059 (30 μg/ml) or transfected with dominant negative (DN) mutant of MEK1 for 24 h followed by stimulation with RANKL, and the ICAM-1 mRNA expression was measured by using qPCR. F: A549 cells were pretreated for 30 min with SB203580 (10 μM) and SP600125 (3 μM) followed by stimulation with RANKL, and in vitro migration was measured with the Transwell after 24 h. Results are expressed as the mean ± SE. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with RANKL-treated group.

transfection efficiency as monitored by the co-transfected β-galactosidase expression vector.

STATISTICS

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

RESULTS

RANKL AND RANK INTERACTION DIRECTS MIGRATION OF LUNG CANCER CELLS

RANKL has been reported to stimulate the directional migration and invasion of human cancer cells [Armstrong et al., 2008; Heymann et al., 2008]. RANKL-triggered migration in lung cancer cells was examined using the Transwell assay with correction for RANKL-

induced proliferation effects on human lung cancer cells [Fong et al., 2008]. RANKL directed human lung cancer (A549) cell migration (Fig. 1A). In addition, RANKL increased migration of other human lung cancer cells (H928 and H1299; Fig. 1B). Interaction of RANKL with its specific receptor RANK on the surface of cancer cells induces cancer invasion [Chen et al., 2006; Heymann et al., 2008]. Pretreatment of cells with RANKL Ab or OPG (a decoy receptor for RANKL) [Van Campenhout and Golledge, 2009] reduced RANKL-induced cell migration (Fig. 1C). However, transfection of cells with RANK siRNA reduced RANK protein expression (Fig. 1D) and antagonized RANKL-induced migration activity (Fig. 1D). Therefore, the RANKL and RANK interaction plays a very important role in the migration of human lung cancer cells.

RANKL-DIRECTED LUNG CANCER CELL MIGRATION INVOLVES ICAM-1 UP-REGULATION

ICAM-1 expression is significantly increased in human cancer cells [Huang et al., 2004]. We therefore hypothesized that ICAM-1 may be involved in RANKL-directed migration of human lung cancer cells. We used qPCR to analyze RANKL-mediated ICAM-1 mRNA expression in A549 cells (Fig. 2A). Transfection of cells with ICAM-1 siRNA markedly inhibited the RANKL-induced cell migration (Fig. 2B). Pretreatment of cells with RANKL Ab or OPG reduced RANKL-mediated ICAM-1 expression (Fig. 2C). In addition, transfection of cells with RANK siRNA reduced RANKL-induced ICAM-1 expression (Fig. 2D). These data suggest that RANKL-induced cancer migration may occur via activation of ICAM-1.

MEK AND ERK SIGNALING PATHWAYS ARE INVOLVED IN RANKL-MEDIATED ICAM-1 UP-REGULATION AND MIGRATION OF HUMAN LUNG CANCER CELLS

The MEK/ERK signaling pathway can be activated by a variety of growth factors, such as insulin and nerve growth factors [Chen et al., 2007; Knauf and Fagin, 2009]. We thus examined whether RANKL stimulation enhances activation of the MEK/ERK pathway. Western blot analysis showed that stimulation of A549 cells with RANKL led to a significant increase in MEK1/2 phosphorylation (Fig. 3A). RANKL-induced migration of A549 cells was greatly reduced by treatment with MEK inhibitors (PD98059 and U0126; Fig. 3B). In addition, transfection of cells with MEK1 mutant reduced RANKL-induced cell migration (Fig. 3C). The MEK inhibitors (PD98059 and U0126) and the MEK1 mutant also inhibited the RANKL-induced ICAM-1 up-regulation (Fig. 3D,E).

To examine the crucial role of MEK/ERK in cancer migration and ICAM-1 up-regulation, we evaluated ERK phosphorylation in response to RANKL incubation. Treatment of A549 cells with RANKL resulted in significant phosphorylation of ERK (Fig. 4A). Transfection with ERK siRNA or ERK2 mutant antagonized the RANKL-induced migration activity and ICAM-1 expression in A549 cells (Fig. 4B,C). To examine whether the other MAPKs (p38 and JNK) are involved in RANKL-induced cell migration, p38 and JNK inhibitors were used. Treatment of cells with p38 inhibitor (SB203580) or JNK inhibitor (SP600125) slightly reduced RANKL-mediated cell migration (Fig. 3F). Therefore, p38 and JNK also participate in RANKL-induced cell motility. Taken together, these results indicate that the MEK and ERK pathways are involved in RANKL-induced migration activity and ICAM-1 up-regulation in human lung cancer cells.

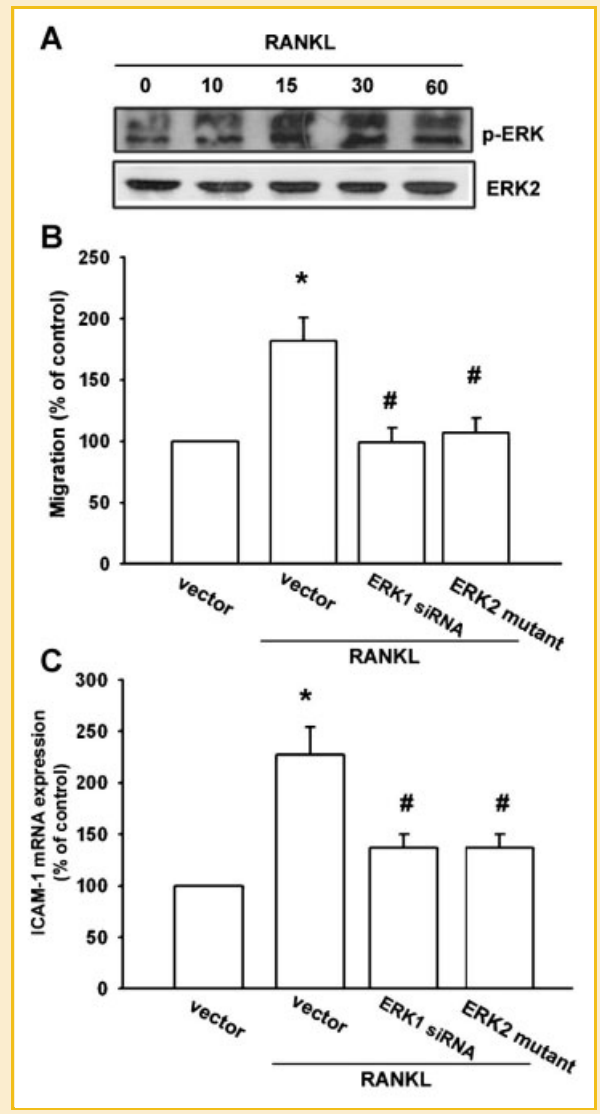

Fig. 4. ERK is involved in RANKL-induced migration and ICAM-1 up-regulation in human lung cancer cells. A: A549 cells were incubated with RANKL for indicated time intervals, and ERK phophorylation was examined by Western blot analysis. B: A549 cells were transfected with ERK1 siRNA or ERK2 mutant for 24 h followed by stimulation with RANKL, and in vitro migration was measured with the Transwell after 24 h. C: A549 cells were transfected with ERK1 siRNA or ERK2 mutant for 24 h followed by stimulation with RANKL, and the ICAM-1 mRNA expression was measured using qPCR. Results are expressed as the mean ± SE. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with RANKL-treated group.

THE NF-κB SIGNALING PATHWAY IS INVOLVED IN RANKL-MEDIATED ICAM-1 UP-REGULATION AND MIGRATION ACTIVITY

As previously mentioned, NF-κB activation is necessary for the migration and invasion of human cancer cells [Fong et al., 2008; Yeh et al., 2008]. To examine whether NF-κB activation is involved in RANKL-induced cancer migration, an NF-κB inhibitor, PDTC, was

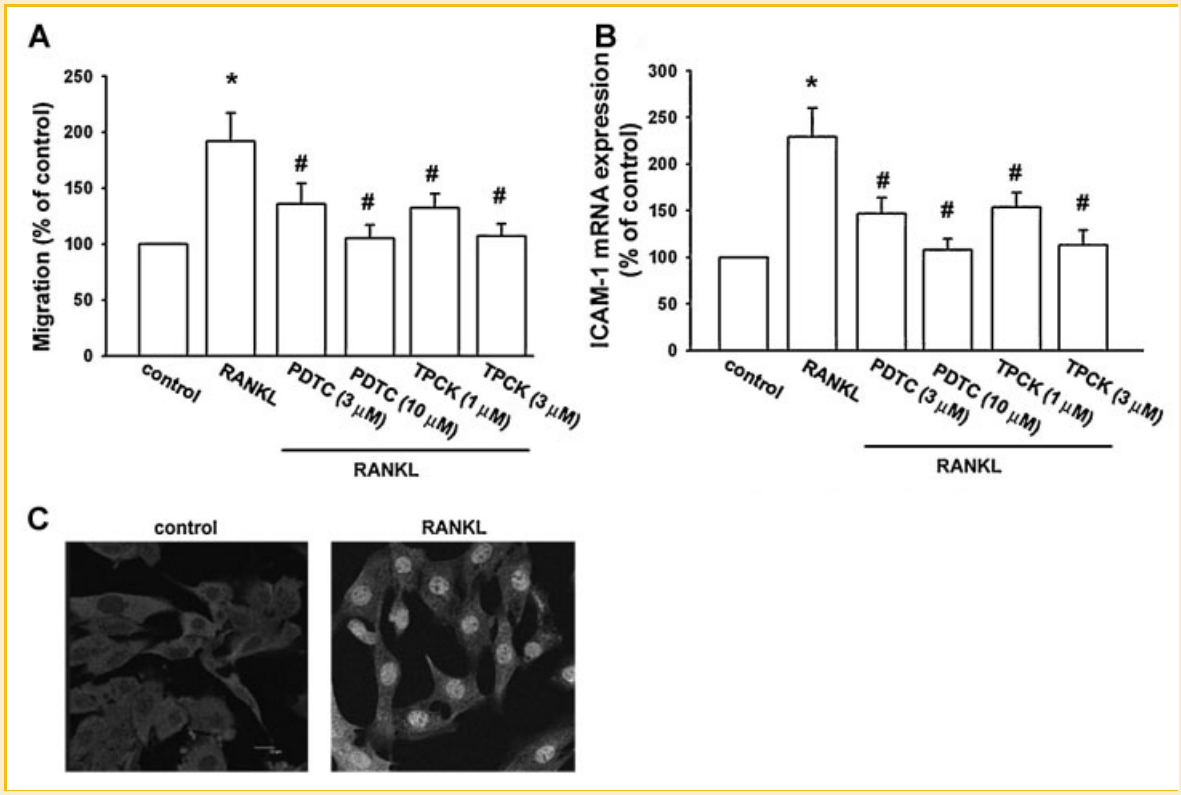

Fig. 5. RANKL induces cells migration and ICAM-1 up-regulation through NF-κB. A: A549 cells were pretreated for 30 min with PDTC (3, 10 μM) or TPCK (1, 3 μM) followed by stimulation with RANKL (30 ng/ml), and in vitro migration was measured with the Transwell after 24 h. B: A549 cells were pretreated for 30 min with PDTC (3, 10 μM) or TPCK (1, 3 μM) followed by stimulation with RANKL (30 ng/ml) for 24 h, and ICAM-1 mRNA expression was measured by qPCR. C: Cells were stimulated stimulation with RANKL for 120 min, and p65 immunofluorescence staining was examined. Results are expressed as the mean ± SE. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with RANKL-treated group.

used. Pretreatment of A549 cells with PDTC [(3, 10 μM)] inhibited the RANKL-induced lung cancer cell migration. Furthermore, A549 cells pretreated with TPCK [(1, 3 μM)], an IκB protease inhibitor, also showed reduced RANKL-induced cancer cell migration (Fig. 5A). In addition, treatment of cells with PDTC or TPCK antagonized RANKL-induced expression of ICAM-1 (Fig. 5B). Immunofluorescence staining showed that stimulation of cells with RANKL increased p65 translocation into the nucleus (Fig. 5C).

p65 Ser^{536} phosphorylation increases NF-κB transactivation [Madrid et al., 2001; Viatour et al., 2005]; thus, the antibody specific for phosphorylated p65 Ser^{536} was used to examine p65 phosphorylation in response to RANKL. Treatment of A549 cells with RANKL for various time intervals resulted in p65 Ser^{536} phosphorylation (Fig. 6A). We next investigated whether p65 binds to the NF-κB element on the ICAM-1 promoter after RANKL stimulation. The recruitment of p65 to the ICAM-1 promoter (−346 to −24) in vivo was assessed using the chromatin immunoprecipitation assay [Huang and Chen, 2005]. In vivo binding of p65, but not no-antibody control or non-immune antibody control, to the NF-κB element of the ICAM-1 promoter occurred after RANKL stimulation. (Fig. 6B, left panel). The binding of p65 to the NF-κB element in response to RANKL was attenuated by OPG and PD98059 (Fig. 6B, right panel). To directly determine NF-κB activation after RANKL treatment, cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. RANKL treatment of A549 cells for 24 h increased κB-luciferase activity. In addition, RANK Ab, OPG, PD98059, and U0126 antagonized the RANKL-induced κB-luciferase activity (Fig. 6C). Co-transfection of cells with RANK siRNA or the MEK or ERK2 mutant also reduced RANKL-induced κB-luciferase activity (Fig. 6D). Taken together, these data suggest that activation of RANK, MEK, and ERK are required for RANKL-induced NF-κB activation in human lung cancer cells.

DISCUSSION

Lung cancer is by far the most common cause of cancer-related death in the world [Greenlee et al., 2001]. This high mortality is probably attributable to early metastasis, principally through the spreading of malignant cells, especially NSCLC cells, to many tissues including bone [Gupta and Massague, 2006]. Therefore, early detection of cancer, prevention of metastasis, and an understanding of the mechanism of metastatic activity of cancer cells are all vitally important to reduce lung cancer deaths. To metastasize, cancer cells must overcome several barriers. Several discrete steps are discernible in the biological cascade of metastasis: loss of cellular adhesion, increased motility and invasiveness, entry into and survival in circulation, spreading into new tissue, and eventual colonization of a distant site. We hypothesized that RANKL and its receptor, RANK, help to direct the migration of lung cancer cells. We

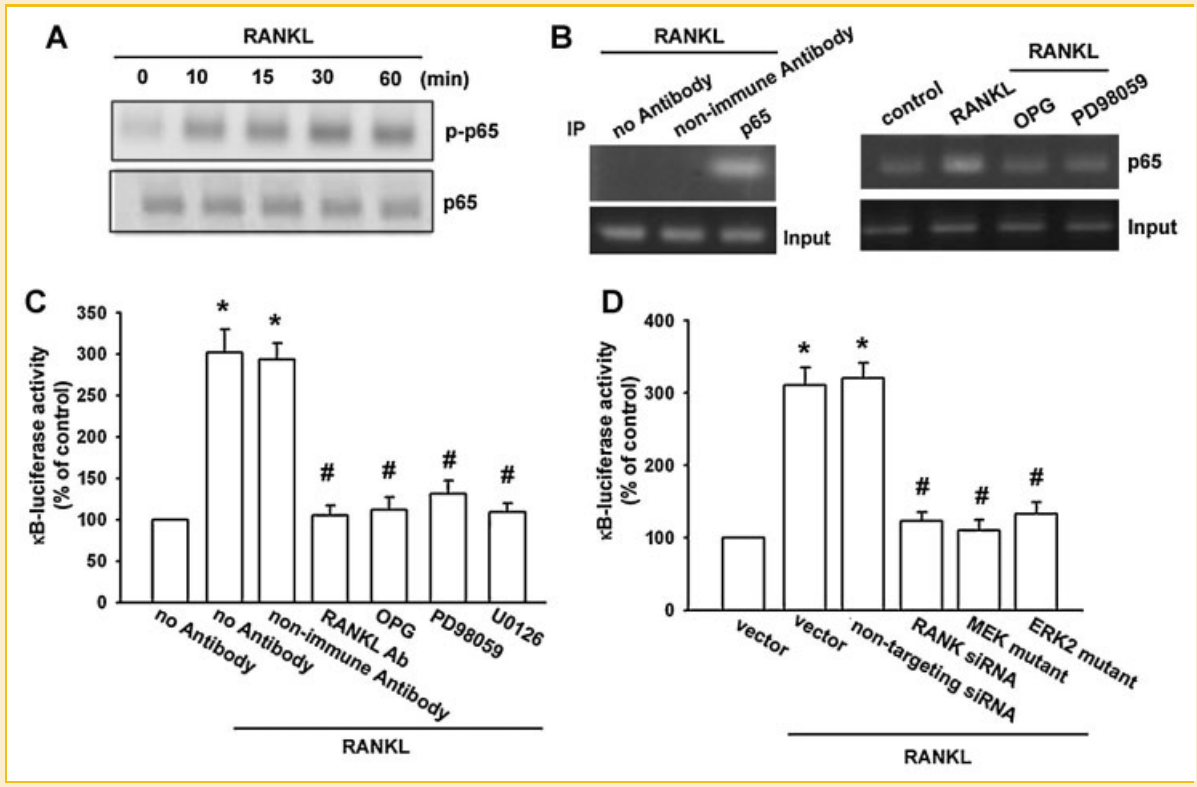

Fig. 6. MEK/ERK pathway is involved in RANKL-mediated NF-κB activation and ICAM-1 expression. A: A549 cells were incubated with RANKL for indicated time intervals, and p65 phophorylation was examined by Western blot analysis. (B; left panel) Cells were stimulated with RANKL for 120 min, and the chromatin immunoprecipitation assay was then performed. Chromatin was immunoprecipitated with no-antibody, non-immune antibody or p65. One percentage of the precipitated chromatin was assayed to verify equal loading (input). (B; right panel) Cells were pretreated with OPG (30 ng/ml) and PD98059 (30 μg/ml) then stimulated with RANKL for 120 min, and the chromatin immunoprecipitation assay was then performed. Chromatin was immunoprecipitated with anti-p65. One percentage of the precipitated chromatin was assayed to verify equal loading (input). C: Cells were pretreated with RANKL Ab (10 μg/ml), OPG (30 ng/ml), PD98059 (30 μg/ml) and U0126 (30 μg/ml) for 30 min or transfected with RANK siRNA and mutant of MEK or ERK (D) before exposure to RANKL. NF-κB luciferase activity was measured, and the results were normalized to the β-galactosidase activity and expressed as the mean ± SE for three independent experiments performed in triplicate. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with RANKL-treated group.

found that RANKL increased the migration of human lung cancer cells via activation of the RANK, MEK, ERK, and NF-κB pathways.

RANKL and RANK play a key role in osteoclastogenesis and tumor metastasis [Kearns et al., 2008]. The consequences of expression of RANKL and RANK in human lung cancer cells are not well understood. In this study, we found that pretreatment with RANKL Ab or OPG reduced RANKL-induced cell migration. This was further confirmed by the result that RANK siRNA inhibited the enhancement of migration by RANKL. Therefore, the RANKL and RANK interaction mediated migration activity in human lung cancer cells.

A variety of growth factors stimulate the expression of ICAM-1 via signal-transduction pathways that converge to activate the NF-κB complex of transcription factors [Huang et al., 2004]. The MEK/ERK pathway is a major cascade mediating activation of the NF-κB signaling pathway in human cancer cells [Kapur et al., 2003]. We found RANKL-enhanced MEK phosphorylation in human lung cancer cells. Pretreatment of cells with the MEK inhibitor U0126 or PD98059 antagonized the increase in migration and ICAM-1 expression that was induced by RANKL stimulation. This was further confirmed by the finding that the dominant-negative MEK1 mutant inhibited the enhancement of migration by RANKL. Moreover, we also found that RANKL activated ERK phosphorylation, the ERK2 mutant inhibited RANKL-mediated migration activity and ICAM-1 up-regulation. Our data indicate that MEK/ERK may play an important role in the expression of ICAM-1 and migration of human lung cancer cells.

There are several binding sites for a number of transcription factors including AP-1, NF-κB, CCAAT/enhancer-binding protein, and SP in the human ICAM-1 promoter [van de Stolpe and van der Saag, 1996]. The results of this study showed that NF-κB activation contributed to RANKL-induced migration and ICAM-1 production in human lung cancer cells. The NF-κB sequence binds to members of the p65 and p50 families of transcription factors. p65 is phosphorylated at Ser^{536} by a variety of kinases through various signaling pathways, and this enhances the p65 transactivation potential. TNF-α induces rapid p65 phosphorylation at Ser^{536} through IKKs, resulting in increased transcriptional activity of p65 [Sakurai et al., 1999]. In our study, RANKL increased the phosphorylation of p65. Furthermore, RANKL also increased the binding of p65 to the NF-κB element on the ICAM-1 promoter, as shown by the chromatin immunoprecipitation assay. Binding of p65 to the NF-κB element was attenuated by OPG and PD98059. Our data indicate that the RANK/MEK/ERK and NF-κB pathways might play an important role in the expression of ICAM-1 and migration of human lung cancer cells.

The prognosis of patients with metastatic lung cancer is generally considered very poor; hence, preventing lung cancer metastasis is important for survival. Our study indicates that RANKL increases the expression of ICAM-1 via the RANK, MEK, ERK, and NF-κB-dependent pathways and enhances migration of human lung cancer cells. Furthermore, the discovery of a RANKL/RANK-mediated signaling pathway helps elucidate the mechanism of human lung cancer metastasis and may lead us to develop effective therapies.

ACKNOWLEDGMENTS

This study was supported by grants from the Taiwan Department of Health, China Medical University Hospital Cancer Research of Excellence (DOH99-TD-C-111-005) and from the Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH99-TD-B-111-004). We thank Dr. W. M. Fu for providing the MEK1 mutant and Dr. M. Cobb for providing the ERK2 mutant.

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