

JNK1 Differentially Regulates Osteopontin-induced Nuclear Factor-inducing Kinase/MEKK1-dependent Activating Protein-1-mediated Promatrix Metalloproteinase-9 Activation*

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We have recently demonstrated that nuclear factor-inducing kinase (NIK) plays a crucial role in osteopontin (OPN)-induced mitogen-activated protein kinase/IKK β kinase-dependent nuclear factor κ B (NF κ B)-mediated promatrix metalloproteinase-9 activation (Rangaswami, H., Bulbule, A., and Kundu, G. C. (2004) *J. Biol. Chem.* 279, 38921–38935). However, the molecular mechanism(s) by which OPN regulates NIK/MEKK1-dependent activating protein-1 (AP-1)-mediated promatrix metalloproteinase-9 activation and whether JNK1 plays any role in regulating both these pathways that control the cell motility are not well defined. Here we report that OPN induces α v β 3 integrin-mediated MEKK1 phosphorylation and MEKK1-dependent JNK1 phosphorylation and activation. Overexpression of NIK enhances OPN-induced c-Jun expression, whereas overexpressed NIK had no role in OPN-induced JNK1 phosphorylation and activation. Sustained activation of JNK1 by overexpression of wild type but not kinase negative MEKK1 resulted in suppression of ERK1/2 activation. But this did not affect the OPN-induced NIK-dependent ERK1/2 activation. OPN stimulated both NIK and MEKK1-dependent c-Jun expression, leading to AP-1 activation, whereas NIK-dependent AP-1 activation is independent of JNK1. OPN also enhanced JNK1-dependent/independent AP-1-mediated urokinase type plasminogen activator (uPA) secretion, uPA-dependent promatrix metalloproteinase-9 (MMP-9) activation, cell motility, and invasion. OPN stimulates tumor growth, and the levels of c-Jun, AP-1, urokinase type plasminogen activator, and MMP-9 were higher in OPN-induced tumor compared with control. To our knowledge this is first report that OPN induces NIK/MEKK1-mediated JNK1-dependent/independent AP-1-mediated pro-MMP-9 activation and regulates the negative cross-talk between NIK/ERK1/2 and MEKK1/JNK1 pathways that ultimately controls the cell motility, invasiveness, and tumor growth.

Osteopontin (OPN),¹ a non-collagenous, sialic acid-rich and glycosylated phosphoprotein, is a member of the extracellular

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¹ The abbreviations used are: OPN, osteopontin; NIK, nuclear factor-inducing kinase; MEKK1, mitogen-activated protein kinase kinase kinase; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; AP-1, activating protein-1; EMSA, electrophoretic mobility

matrix (ECM) protein family (1, 2). OPN acts both as chemokine and cytokine. It is produced by osteoclast, macrophages, T cells, hematopoietic cells, and vascular smooth muscle cells (3). It has an N-terminal signal sequence, a highly acidic region consisting of nine consecutive aspartic acid residues, and a GRGDS cell adhesion sequence predicted to be flanked by the β -sheet structure (4). This protein has a functional thrombin cleavage site and is a substrate for tissue transglutaminase (2). It binds with several integrins and CD44 variants in an RGD sequence-dependent and -independent manner (5, 6). This protein is involved in normal tissue remodeling process such as bone resorption, angiogenesis, wound healing, and tissue injury as well as certain diseases such as tumorigenesis, restenosis, atherosclerosis, and autoimmune diseases (6–8). OPN expression is up-regulated in several cancers and is reported to associate with tumor progression and metastasis (9–11). OPN regulates cell adhesion, cell migration, ECM-invasion, and cell proliferation by interacting with its receptor α v β 3 integrin in various cell types (6). Previous data indicated that OPN induces MT1-MMP-mediated pro-matrix metalloproteinase-2 (pro-MMP-2) activation and urokinase type plasminogen activator (uPA)-mediated pro-matrix metalloproteinase-9 (pro-MMP-9) activation, cell motility, ECM invasion, and tumor growth (12–16).

Integrins are non-covalently associated, heterodimeric, cell-surface glycoproteins with α - and β -subunits. The various combinations of the α - and β -subunits form integrin dimers with diverse ligand specificity and biological activities. The interaction of cell surface integrin with ECM proteins can lead to the regulation of cell growth, differentiation, adhesion, and migration.

MEKK1, a member of the mitogen-activated protein 3-kinase family, is a mammalian serine/threonine protein kinase initially identified on the basis of its homology with STE11 that activates the pheromone-responsive mitogen-activated protein kinase cascade in yeast (17). Previous data indicated that overexpression of constitutively active forms of MEKK1 leads to JNK activation via phosphorylation of its upstream kinase, mitogen-activated protein kinase kinase 4 (18). The data also showed that MEKK1 has the ability to activate ERK, but its effect is less potent (19). These results suggest that MEKK1 is an upstream kinase in the mitogen-activated protein kinase cascade. Nuclear factor-inducing kinase (NIK) is another member of the mitogen-activated protein 3-kinase family that has been implicated in NF κ B activation. Few reports indicate that NIK may also be involved in the regulation of transcription factor, AP-1, as its activation leads to the induction of c-Fos

shift assay; DTT, dithiothreitol; Luc, luciferase; ECM, extracellular matrix; uPA, urokinase type plasminogen activator; MMP-9, matrix metalloproteinase-9; IKK, IKK kinase complex; wt, wild type; dn, dominant negative; mut, mutant.

that associates with c-Jun to form an AP-1 heterodimeric complex that can promote targeted gene expression (20). However, the molecular mechanism by which OPN regulates NIK and MEKK1-mediated AP-1 transactivation and whether JNK is involved in both these pathways is not clearly understood. Various mitogen-activated protein kinase cascades (e.g. ERK1/2, JNK, p38) are often portrayed as linear cascades, and indications of cross-talk between the various cascades are limited (21, 22). In this respect, the present study also examines whether any cross-talk exists between OPN-induced NIK/ERK and MEKK1/JNK-signaling pathways.

uPA is a member of serine protease family that interacts with the uPA receptor and facilitates the conversion of inert zymogen plasminogen into widely acting serine protease plasmin (23). Plasmin regulates cell invasion by degrading matrix proteins such as type IV collagen, gelatin, fibronectin, and laminin or indirectly by activating MMPs (24, 25). It is established that uPA plays a significant role in tumor growth and metastasis (26–28). It is regulated at the transcriptional level by a number of transcription factors. AP-1 transcription factor duplex also plays a major role in the regulation of uPA expression through binding to its promoter (29). However, the molecular mechanism by which OPN regulates NIK and MEKK1-mediated JNK-dependent/independent AP-1 activation and uPA secretion in murine melanoma (B16F10) cells is not well defined.

MMPs are ECM degrading enzymes that play a critical role in embryogenesis, tissue remodeling, inflammation, and angiogenesis (30). We have recently reported that OPN induces NF κ B-mediated pro-MMP-2 activation through I κ B α /IKK signaling pathways (12, 13). MMP-9, also referred to as type IV collagenase or gelatinase B, efficiently degrades native type IV and V collagens, fibronectin, ectactin, and elastin and, hence, plays a major role in invasion, tumor growth, and metastasis (31–34). The regulation of activation of MMP-9 is more complex than most of the other MMPs because most of the cells do not express a constitutively active form of MMP-9, but its activity is induced by different stimuli depending on cell types (35–37), thereby contributing to the specific pathological events. MMP-9 is not only associated with invasion and metastasis but also has been implicated in angiogenesis, rheumatoid arthritis, retinopathy, and vascular stenosis and, hence, is considered to be prioritized therapeutic target (38). However the molecular mechanism by which OPN regulates AP-1-mediated pro-MMP-9 activation and controls cell migration and tumor growth is not well defined.

In this paper we have demonstrated that OPN induces α v β 3 integrin-mediated NIK- and MEKK1-dependent c-Jun expression, leading to AP-1 activation and uPA secretion in B16F10 cells. This OPN-induced MEKK1- and NIK-mediated AP-1 transactivation occurs through both JNK-dependent and -independent pathways. OPN also induces a negative cross-talk between NIK/ERK and MEKK1/JNK1 pathways. Moreover, OPN also induces uPA secretion and uPA-dependent pro-MMP-9 activation, cell motility, invasion, and tumor growth. Taken together, these data demonstrated that OPN induces α v β 3 integrin-mediated NIK and MEKK1 kinase activities that ultimately enhance c-Jun expression through JNK-dependent and -independent pathways. OPN regulates cross-talk between JNK and ERK that leads to the induction of uPA secretion and uPA-dependent pro-MMP-9 activation, cell motility, invasion, and tumor growth.

EXPERIMENTAL PROCEDURES

Materials—The rabbit polyclonal anti-NIK, anti-MEKK1, anti-IKK α / β , anti-JNK1, anti-c-Jun, anti-ERK1/2, anti-uPA, anti-MMP-9 and anti-actin, mouse monoclonal anti-phospho-ERK1/2, anti-phospho-

tyrosine antibodies, recombinant MEK-1, p42 MAPK, and c-Jun proteins were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-MMP-9 was from Oncogene. Mouse monoclonal anti-human α v β 3 integrin antibody was from Chemicon International. Lipofectamine Plus, GRGDSP, and GRGESP were obtained from Invitrogen. Mouse monoclonal anti-phosphoserine detection kit and SP600125 were from Calbiochem. Myelin basic protein was from Sigma. The dual luciferase reporter assay system and AP-1 consensus oligonucleotide were purchased from Promega. Boyden type cell migration chambers were obtained from Corning, and BioCoat Matrigel™ invasion chambers were from Collaborative Biomedical. The [γ -³²P]ATP was purchased from the Board of Radiation and Isotope Technology (Hyderabad, India). The human OPN was purified from milk as described previously and used throughout these studies (12). The nude mice (NMRI, nu/nu) were from National Institute of Virology (Pune, India). All other chemicals were of analytical grade.

Cell Culture—The B16F10 cells were obtained from American Type Culture Collection (Manassas, VA). These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Plasmids and DNA Transfection—The wild type NIK (wt pcDNA NIK) and kinase-negative NIK (mut pcDNA NIK, NIK-K429A/K430A) in pcDNA3 were generous gifts from Prof. David Wallach (Weizmann Institute of Science, Rehovot, Israel). The wild type and kinase negative constructs of MEKK1 (pcDNA3 MEKK1 and pcDNA3 FlagMEKK1 K432M) were kind gifts from Prof. Tom Maniatis (Harvard University, Cambridge). The wild type c-Jun in pRJB10B and dominant negative c-Jun in pELFIN were gifts from Dr. Jalam (Ochsner Clinic Foundation, New Orleans, LA). The wild type and dominant negative forms of JNK1 in pcDNA3 were kind gifts from Dr. Roger Davis (University of Massachusetts Medical School, Worcester, MA). The luciferase reporter construct (pAP-1-Luc) containing seven tandem repeats of the AP-1 binding site was from Stratagene. The B16F10 cells were split 12 h before transfection in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. These cells were transiently transfected with cDNA using Lipofectamine Plus according to manufacturer's instructions as described (Invitrogen). These transfected cells were used for JNK1 and ERK1/2 phosphorylation and kinase assay, c-Jun expression, AP-1-DNA binding, AP-1 luciferase reporter gene assay, and detection of MMP-9 by zymography and Western blot, uPA secretion by Western blot, cell migration, chemoinvasion, and *in vivo* experiments.

Western Blot Analysis—To ascertain the role of MEKK1 and JNK-1 in OPN-induced uPA secretion, the cells were transfected with wild type and kinase negative MEKK1 or wild type and dominant negative JNK-1 or pretreated with 50 μ M SP600125 (JNK-1 inhibitor) and then treated with 5 μ M OPN for 24 h. To examine whether c-Jun plays any role in OPN-induced uPA secretion, cells were transfected with wild type and dominant negative c-Jun and then treated with OPN. The cell lysates containing equal amount of total proteins were subjected to Western blot analysis using rabbit polyclonal anti-uPA antibody and detected by ECL detection system (Amersham Biosciences). As loading controls, the expression of actin was also detected by reprobating the blots with rabbit anti-actin antibody.

Immunoprecipitation—To examine the effect of OPN in regulation of MEKK1 phosphorylation, cells were treated with 5 μ M OPN at 37 °C for 0–60 min. In separate experiments, cells were pretreated with anti- α v β 3 integrin antibody (20 μ g/ml), GRGDSP or GRGESP peptide (10 μ M) and then treated with 5 μ M OPN. The cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride), and cell lysates containing equal amount of total proteins were immunoprecipitated with rabbit polyclonal anti-MEKK1 antibody. The immunocomplexes were analyzed by Western blot using mouse monoclonal anti-phosphoserine antibody. As loading control, same blots were reprobated with rabbit polyclonal anti-MEKK1 antibody.

To delineate the role of OPN in regulation of JNK-1 phosphorylation, the cells were treated with 5 μ M OPN for 0–90 min at 37 °C. In separate experiments cells were pretreated with anti- α v β 3 integrin antibody (20 μ g/ml), GRGDSP or GRGESP peptide (10 μ M) and then treated with 5 μ M OPN. Cell lysates were immunoprecipitated with rabbit polyclonal anti-JNK-1 antibody. The immunocomplexes were analyzed by Western blot using mouse monoclonal anti-phosphotyrosine antibody. The same blots were reprobated with rabbit anti-JNK-1 antibody as loading control.

To detect whether NIK and MEKK1 are involved in the regulation of OPN induced JNK-1 phosphorylation, cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1

and then treated with 5 μ M OPN for 15 min. Cell lysates were immunoprecipitated with rabbit polyclonal anti-JNK-1 antibody and analyzed by Western blot using anti-phosphotyrosine antibody. The same blots were reprobed with anti-JNK1 antibody.

Nuclear Extracts and Western Blot—To check the level of c-Jun expression in the nucleus, cells were treated with 5 μ M OPN for 0–4 h at 37 °C. In separate experiments, cells were pretreated with anti- α v β 3 integrin antibody (20 μ g/ml), GRGDSP or GRGESP peptide (10 μ M) or transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 and then treated with 5 μ M OPN. The nuclear extracts were prepared as described (16). Briefly, cells were incubated in hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) and allowed to swell on ice for 10 min. Cells were homogenized in a Dounce homogenizer. The nuclei were separated by spinning at 3300 \times g for 5 min at 4 °C. The nuclear pellet was extracted in nuclear extraction buffer (20 mM Hepes (pH 7.9), 0.4 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol) and centrifuged at 12,000 \times g for 30 min. The supernatant was used as nuclear extract. The protein concentration was measured by the Bio-Rad protein assay. The nuclear extracts were resolved by SDS-PAGE, and the level of c-Jun was detected by Western blot using rabbit polyclonal anti-c-Jun antibody.

EMSA—To check whether NIK and MEKK1 play any role in OPN-induced AP-1-DNA binding, cells were either treated with 5 μ M OPN or transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 and then treated with OPN for 1 h at 37 °C. The nuclear extracts were prepared as described earlier (16). To investigate whether JNK1 is involved in OPN-induced AP-1-DNA binding and whether it is regulated by NIK, cells were either pretreated with SP600125 (JNK-1 inhibitor) or transfected with wild type NIK and then treated with OPN. The nuclear extracts (10 μ g) were incubated with 16 fmol of ³²P-labeled double-stranded AP-1 oligonucleotide (5'-CGC TTG ATG ACT CAG CCG GAA-3') in binding buffer (25 mM Hepes (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, 50 mM NaCl) containing 2 μ g of poly(dI-dC). The DNA-protein complex was resolved on a native polyacrylamide gel and analyzed by autoradiography. For supershift assay, the OPN-treated nuclear extracts were incubated with anti-c-Jun antibody for 30 min at room temperature and analyzed by EMSA.

AP-1 Luciferase Reporter Gene Assay—The semi-confluent cells grown in 24-well plates were transiently transfected with a luciferase reporter construct (pAP-1-Luc) containing seven tandem repeats of the AP-1 binding site using Lipofectamine Plus reagent (Invitrogen). In separate experiments, cells were individually transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 or wild type and dn JNK1 along with AP-1-Luc. The transfection efficiency was normalized by cotransfecting the cells with pRL vector (Promega) containing a full-length Renilla luciferase gene under the control of a constitutive promoter. After 24 h of transfection, cells were either pretreated with SP600125 for 1 h and/or treated with 5 μ M OPN for 6 h. Cells were harvested in passive lysis buffer (Promega). The luciferase activities were measured by luminometer (Lab Systems) using the dual luciferase assay system according to the manufacturer's instructions (Promega). Changes in luciferase activity with respect to control were calculated.

Zymography Experiments—The gelatinolytic activity was measured as described (12, 13). To ascertain the role of MEKK1 and JNK1 on OPN-induced pro-MMP-9 activation, the cells were transfected with wild type and kinase negative MEKK1 or wild type and dn JNK1 or pretreated with SP600125 (0–50 μ M) for 1 h and then treated with 5 μ M OPN. The conditioned medium was collected, and the samples containing equal amount of total proteins were mixed with sample buffer in the absence of reducing agent and loaded onto zymography-SDS-PAGE-containing gelatin (0.5 mg/ml) as described (12, 13). The gels were incubated in incubation buffer (50 mM Tris-HCl (pH 7.5) containing 100 mM CaCl₂, 1 μ M ZnCl₂, 1% (v/v) Triton-X100, and 0.02% (w/v) NaN₃) for 16 h. The gels were stained with Coomassie Blue and destained. Negative staining showed the zones of gelatinolytic activity.

In Vitro Kinase Assay—To examine the effect of OPN on JNK activity, the semiconfluent cells were treated with 5 μ M OPN for 15 min at 37 °C. To investigate the role of NIK and MEKK1 in OPN-induced JNK activity, in separate experiments the cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 in presence of Lipofectamine plus and then treated with 5 μ M OPN. The cells were lysed in kinase assay lysis buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β -glycerophosphate, 10

mM NaF, 10 mM *p*-nitrophenyl phosphate, 300 μ M Na₃VO₄, 1 mM benzamide, 2 μ M phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1 mM DTT, and 0.25% Nonidet P-40). The supernatant was obtained by centrifugation at 12,000 \times g for 10 min at 4 °C. The cell lysates (300 μ g) containing equal amount of total proteins were immunoprecipitated with rabbit polyclonal anti-JNK1 antibody. Half of the immunoprecipitated samples were incubated with recombinant c-Jun as substrate in kinase assay buffer (20 mM Hepes (pH 7.7), 2 mM MgCl₂, 10 mM β -glycerophosphate, 10 mM NaF, 10 mM *p*-nitrophenyl phosphate, 300 μ M Na₃VO₄, 1 mM benzamide, 2 μ M phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, and 1 mM DTT) containing 10 μ M ATP and 3 μ Ci of [γ -³²P]ATP at 30 °C. The kinase reactions were stopped by the addition of SDS sample buffer. The samples were resolved by SDS-PAGE, dried, and autoradiographed. The remaining half of the immunoprecipitated samples were subjected to SDS-PAGE and analyzed by Western blot using anti-JNK1 antibody. The levels of MEKK1 and NIK expressions in the transfected cell lysates containing equal amount of total proteins were detected by Western blot using anti-MEKK1 or anti-NIK antibody.

To analyze the effect of overexpressed MEKK1 on OPN-induced ERK1/2 activity, cells were transfected with wild type and kinase negative MEKK1 and then treated with OPN. In separate experiments, the wild type MEKK1-transfected cells were also cotransfected with wild type or dominant negative JNK-1 or treated with 20 μ M SP600125 (JNK1 inhibitor) and then treated with OPN. The cells lysates containing equal amount of total proteins were immunoprecipitated with anti-ERK1/2 antibody. Half of the immunocomplexes were incubated with 2 μ g of myelin basic protein in kinase assay buffer supplemented with 10 μ M ATP and 3 μ Ci of [γ -³²P]ATP at 30 °C for 10 min. The samples were resolved on SDS-PAGE and autoradiographed. The remaining half of the immunoprecipitated samples were analyzed by Western blot using anti-ERK1/2 antibody.

NIK-coupled Kinase Assay—NIK-coupled kinase activity was assayed as described previously (39). Briefly, cells were treated with 5 μ M OPN. In separate experiments, cells were transfected with wild type NIK and then treated with OPN. In other experiments the wild type NIK-transfected cells were cotransfected with wild type and kinase negative MEKK1 and then treated with OPN. Cell lysates were immunoprecipitated with anti-NIK antibody. Half of the immunocomplexes were incubated with 0.5 μ g of recombinant MEK-1 protein in kinase assay buffer containing 100 μ M ATP, 10 μ Ci of [γ -³²P]ATP for 20 min at 30 °C. After that, 2 μ g of recombinant kinase inactive ERK (p42) protein was added to the reaction mixture, and the samples were incubated for an additional 30 min at 30 °C. The samples were resolved on SDS-PAGE and autoradiographed. The remaining half of the immunoprecipitated samples was analyzed by Western blot using anti-NIK antibody. The NIK activity was also assayed under the same conditions using IKK as substrate as described previously (16).

Cell Migration and Chemoinvasion Assays—The migration and invasion assays were conducted using non-coated or Matrigel™-coated Transwell cell culture chambers according to the standard procedure as described previously (12–16). Briefly, cells were transfected with wild type and kinase negative MEKK1 or wild type and dn JNK1 or wild type and dn c-Jun or pretreated with SP600125. In separate experiments cells were transfected with wild type and kinase negative NIK followed by treatment with SP600125. The transfected or treated cells were harvested with trypsin-EDTA and centrifuged at 800 \times g for 10 min. The cell suspension (5 \times 10⁵ cells/well) was added to the upper chamber of the uncoated or Matrigel™-coated prehydrated polycarbonate membrane filter. The lower chamber was filled with fibroblast condition medium, which acted as a chemoattractant. Purified OPN (5 μ M) was added to the upper chamber. The cells were incubated in a humidified incubator in 5% CO₂ and 95% air at 37 °C for 16 h. The non-migrated cells and/or the Matrigel™ from the upper side of the filter were scraped and removed using moist cotton swab. The migrated or invaded cells in the reverse side of the filter were fixed with methanol and stained with Giemsa. The migrated or invaded cells on the filter were counted under an inverted microscope (Olympus). The experiments were repeated in triplicate. Preimmune IgG served as nonspecific control.

In Vivo Tumorigenicity Experiments—The tumorigenicity experiments were performed as described previously (12, 13, 16). The cells were treated in the absence or presence of purified human OPN (10 μ M) at 37 °C for 20 h. In separate experiments cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 in the presence of Lipofectamine plus and then treated with 5 μ M OPN. After that, the cells (5 \times 10⁶/0.2 ml) were detached and

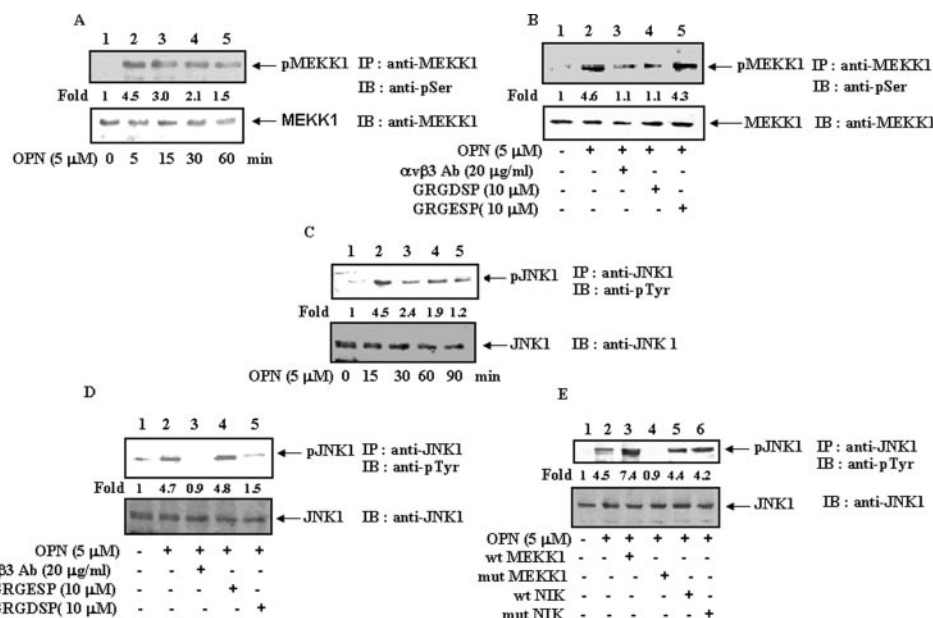


FIG. 1. Panel A, OPN stimulates MEKK1 phosphorylation (pMEKK1). B16F10 cells were treated with 5 μ M OPN for 0–60 min. Cell lysates were immunoprecipitated (IP) with anti-MEKK1 antibody and analyzed by Western blot (IB) using anti-phosphoserine (p-Ser) antibody (Ab) (upper panel A, lanes 1–5), and the same blots were reprobed with anti-MEKK1 antibody (lower panel A). Panel B, OPN induces α v β 3 integrin-mediated MEKK1 phosphorylation. The cells were individually pretreated with anti- α v β 3 integrin antibody, GRGDSP, or GRGESp and then treated with 5 μ M OPN. The cell lysates were immunoprecipitated with anti-MEKK1 antibody and analyzed by Western blot using anti-phosphoserine antibody (upper panel B, lanes 1–5), and same blots were reprobed with anti-MEKK1 antibody (lower panel B). Panels C and D, OPN stimulates α v β 3 integrin-mediated JNK1 phosphorylation (pJNK1). Cells were treated with 5 μ M OPN for 0–90 min or pretreated with anti- α v β 3 integrin antibody, GRGDSP, or GRGESp and then treated with 5 μ M OPN for 15 min. The cell lysates were immunoprecipitated with anti-JNK1 antibody and analyzed by Western blot using anti-phosphotyrosine (p-Tyr) antibody (upper panels C and D, lanes 1–5). The same blots were reprobed with anti-JNK1 antibody (lower panels C and D). Panel E, OPN-induced JNK1 phosphorylation is enhanced by MEKK1 but not by NIK. Cells were transfected with wild type and kinase negative MEKK1 or wild type and kinase negative NIK and then stimulated with OPN. Cell lysates were immunoprecipitated with anti-JNK1 antibody and analyzed by Western blot using anti-phosphotyrosine antibody (upper panel E, lanes 1–6). Same blots were reprobed with anti-JNK1 antibody (lower panel E). All these bands were analyzed densitometrically, and the -fold changes were calculated. The data shown here represent three experiments exhibiting similar effects.

injected subcutaneously into the flanks of male athymic NMRI (nu/nu) mice (6–8 weeks old). Four mice were used in each set of experiments. The mice were kept under specific pathogen-free conditions. OPN (10 μ M) was again injected into the tumor sites twice a week for up to 4 weeks. After 4 weeks the mice were killed, and the tumor weights were measured. The tumor tissues were homogenized and lysed in lysis buffer (50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 1% Nonidet P-40, 15 μ g/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride) and centrifuged at 12,000 \times g for 10 min. The clear supernatants were collected, and the levels of uPA and MMP-9 were detected by Western blot analysis using specific antibodies. To check the level of c-Jun expression and AP-1-DNA binding, the tumor tissues were homogenized in buffer A (10 mM HEPES buffer (pH 7.9) containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10% Nonidet P-40, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 0.5 mg/ml benzamide), and the nuclei was separated by spinning at 13,000 \times g for 10 min at 4 $^{\circ}$ C. The nuclear pellet was extracted in Buffer C (20 mM HEPES buffer (pH 7.9) containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin) for 2 h on ice and centrifuged at 13,000 \times g for 5 min at 4 $^{\circ}$ C. The supernatant was used as nuclear extract. The nuclear extracts were subjected to Western blot analysis using anti-c-Jun antibody. The AP-1-DNA binding in the nuclear extracts was performed by EMSA as described above.

RESULTS

OPN Induces α v β 3 Integrin-dependent MEKK1 and JNK1 Phosphorylations—To investigate the role of OPN on MEKK1 and JNK1 phosphorylations and to demonstrate the involvement of α v β 3 integrin in this activation process, B16F10 cells were treated with 5 μ M OPN at 37 $^{\circ}$ C or pretreated with anti- α v β 3 integrin antibody or RGD/RGE peptide (GRGDSP or GRGESp) and then treated with OPN. The cell lysates were immunoprecipitated with anti-MEKK1 antibody and analyzed by Western blot using anti-phosphoserine antibody. For JNK phosphorylation studies, cell lysates were immunoprecipitated with anti-JNK1 antibody and analyzed by Western blot using

anti-phosphotyrosine antibody. The data revealed that maximum level of OPN-induced MEKK1 and JNK1 phosphorylations occurred at 5 and 15 min, respectively (Fig. 1, upper panels of A and C, lanes 1–5). As loading controls, same blots were reprobed with anti-MEKK1 or anti-JNK1 antibody (lower panels of A and C, lanes 1–5). Pretreatment of cells with anti- α v β 3 integrin antibody or RGD (GRGDSP) but not RGE (GRGESp) peptide suppressed the OPN-induced MEKK1 and JNK1 phosphorylations in these cells (upper panel of B and D, lanes 1–5). Same blots were reprobed with anti-MEKK1 or anti-JNK1 antibody (lower panels of B and D, lanes 1–5). All the bands were quantified by densitometric analysis, and the -fold changes were calculated. These results demonstrated that OPN induces MEKK1 and JNK1 phosphorylations through α v β 3 integrin-mediated pathway.

MEKK1 but Not NIK Is Required for OPN-induced α v β 3 Integrin-mediated JNK1 Phosphorylation—Because we have reported earlier that OPN induces NIK-dependent NF κ B-mediated pro-MMP-9 activation through ERK/IKK-mediated pathways, therefore we sought to examine whether NIK/MEKK1 plays any role in OPN-induced JNK1 phosphorylation in B16F10 cells. Accordingly, cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 and then treated with OPN. Cell lysates were immunoprecipitated with anti-JNK1 antibody and analyzed by Western blot using anti-phosphotyrosine antibody. Wild type MEKK1 enhanced, whereas kinase negative MEKK1 suppressed the OPN-induced JNK phosphorylation (Fig. 1, upper panel of E, lanes 1–4). The data also indicated that OPN-induced JNK1 phosphorylation was unaffected upon transfection of cells with both wild type and kinase negative NIK (lanes 5 and 6). The same blots were reprobed with anti-JNK1 anti-

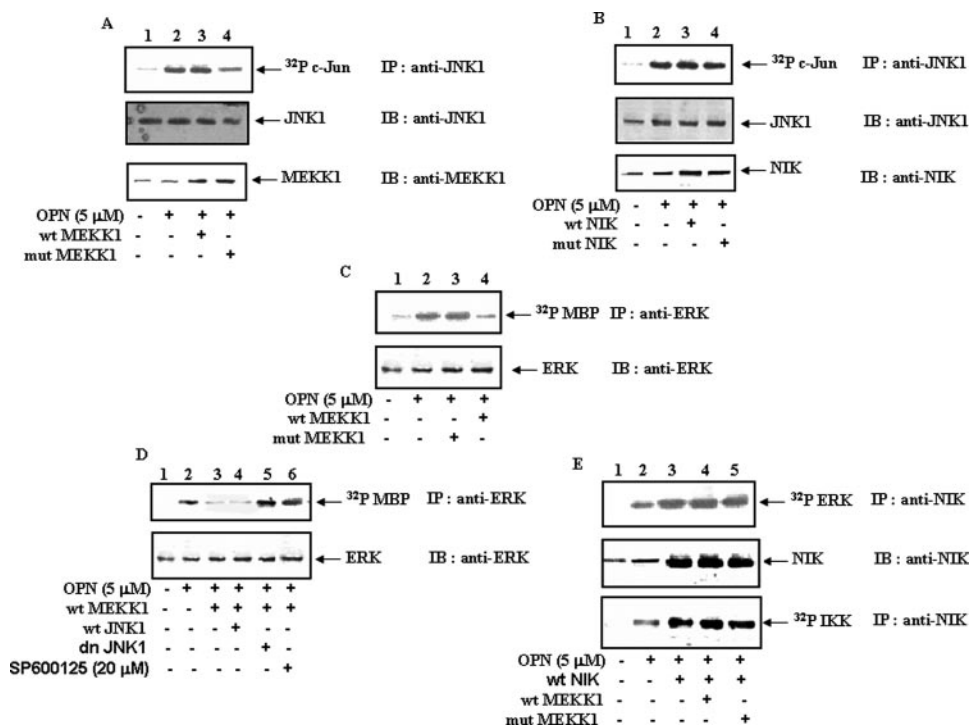


FIG. 2. Panels A and B, OPN induces MEKK1 (panel A)- but not NIK (panel B)-dependent JNK1 activity. Cells were treated with 5 μM OPN or transfected with wild type and kinase negative MEKK1 or wild type and kinase negative NIK and then stimulated with OPN. Cell lysates were immunoprecipitated (IP) with anti-JNK1 antibody, and half of the immunoprecipitated samples were used for JNK kinase assay using recombinant c-Jun as substrate (upper panels A and B, lanes 1–4). The remaining half of the immunoprecipitated samples were immunoblotted (IB) with anti-JNK1 antibody (middle panels A and B, lanes 1–4). The levels of expressions of MEKK1 and NIK in the cell lysates were detected by Western blot using anti-MEKK1 (lower panel A, lanes 1–4) or anti-NIK antibody (lower panel B, lanes 1–4). Panel C, overexpression of MEKK1 attenuates OPN-induced ERK activation. Cells were transfected with wild type and kinase negative MEKK1 and then treated with OPN. Cell lysates were immunoprecipitated with anti-ERK1/2 antibody, and half of the immunoprecipitated samples were used for ERK kinase assay using myelin basic protein as substrate (upper panel C, lanes 1–4). Half of the immunoprecipitated samples were immunoblotted with anti-ERK1/2 antibody (lower panel C, lanes 1–4). Panel D, JNK1 inhibition enhances OPN-induced ERK activation. Cells were cotransfected with wild type MEKK1 along with wild type or dn JNK1 and then treated with OPN. In separate experiments cells were transfected with wild type MEKK1 and treated with SP600125 followed by OPN. Cell lysates were immunoprecipitated with anti-ERK1/2 antibody and used for ERK kinase assay (upper panel D, lanes 1–6). Half of the immunoprecipitated samples were analyzed by Western blot using anti-ERK1/2 antibody (lower panel D). MBP, myelin basic protein. Panel E, OPN-induced NIK-dependent ERK activation is unaffected by overexpression of MEKK1. Cells were cotransfected with wild type NIK along with wild type or kinase negative MEKK1 and then treated with OPN. Cell lysates were immunoprecipitated with anti-NIK antibody and used for NIK coupled kinase assay using MEK and ERK as substrates as described under “Experimental Procedures” (upper panel E, lanes 1–5). Half of the immunoprecipitated samples were immunoblotted with anti-NIK antibody (middle panel E). The NIK activity was also assayed under the same conditions using IKK as substrate (lower panel E, lanes 1–5). The data shown here represent three experiments exhibiting similar effects.

body as loading control (lower panel of E, lanes 1–6). These data suggested that MEKK1 but not NIK plays a crucial role in OPN-induced JNK1 phosphorylation.

MEKK1 but Not NIK Enhances the OPN-induced JNK1 Activity—To ascertain the role of OPN on JNK1 activity, the cells were treated with 5 μM OPN, and the cell lysates were immunoprecipitated with rabbit anti-JNK1 antibody. Half of the immunoprecipitated samples were incubated with recombinant c-Jun as substrate in kinase assay buffer. The samples were resolved by SDS-PAGE and autoradiographed. The radiolabeled, phosphorylated c-Jun-specific band is detected in OPN-treated cells, demonstrating that OPN induces JNK1 activity (Fig. 2, upper panel of A, lane 2). The JNK1 activity is not detected in the untreated cells (lane 1). To further check whether MEKK1 and NIK play any direct role in OPN-induced JNK1 activity, in separate experiments cells were transfected with wild type and kinase negative MEKK1 or wild type and kinase negative NIK and then treated with OPN, and a JNK1 kinase assay was performed. Cells transfected with wild type MEKK1 followed by treatment with OPN showed maximum JNK1 activity (lane 3) compared with untreated cells (lane 1). The cells transfected with kinase negative MEKK1 followed by treatment with OPN showed reduced level of JNK1 activity (lane 4), indicating that the kinase domain of MEKK1 plays a

crucial role in OPN-induced JNK1 activity. The data also indicated that OPN-induced JNK1 activity was unaffected upon overexpression of both wild type and kinase negative NIK (upper panel B, lanes 1–4). The remaining half of the immunoprecipitated samples was analyzed by Western blot using anti-JNK1 antibody (middle panels A and B, lanes 1–4). The levels of MEKK1 and NIK were also analyzed by Western blot using anti-MEKK1 and anti-NIK antibodies, respectively (lower panels A and B, lanes 1–4). These results suggested that MEKK1 but not NIK plays a significant role in modulating OPN-induced JNK activity.

Overexpression of Active MEKK-1 Attenuates OPN-induced ERK1/2 Activation—MEKK-1 functions as a mitogen-activated protein kinase kinase kinase in the JNK pathway; however, several reports have suggested that MEKK-1 may also affect the ERK pathway (21). To determine the effect of MEKK1 on OPN-induced ERK activation, cells were transfected with wild type and kinase negative MEKK1 and then treated with OPN. The cell lysates were immunoprecipitated with anti-ERK1/2 antibody, and kinase activity was measured using myelin basic protein as the substrate. The data indicated that overexpression of wild type MEKK1 almost completely attenuates OPN-induced ERK activation (Fig. 2, upper panel C, lanes 1–4). This abrogation depends on MEKK-1 kinase activity because ERK

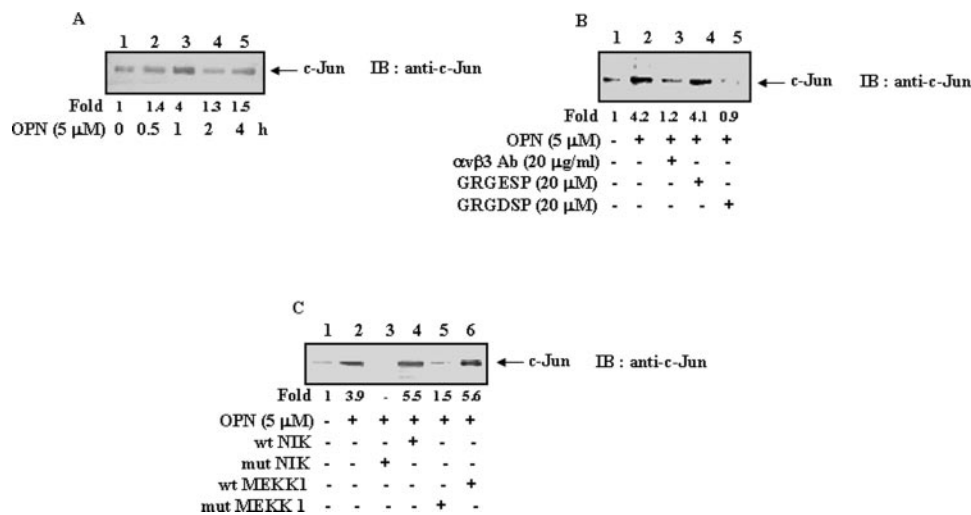


FIG. 3. Panels A and B, OPN enhances $\alpha\beta$ integrin-mediated c-Jun expression. Cells were treated with 5 μM OPN for 0–4 h or pretreated with anti- $\alpha\beta$ integrin antibody, RGD/RGE peptide (GRGDSP or GRGESP), and then stimulated with OPN. The nuclear extracts were prepared as described under “Experimental Procedures.” The level of c-Jun in the nuclear extracts was analyzed by Western blot (IB) using anti-c-Jun antibody (panels A and B, lanes 1–5). Panel C, NIK and MEKK1 play independent roles in OPN-induced c-Jun expression. Cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 and then treated with OPN. The nuclear extracts were prepared and analyzed by Western blot using anti-c-Jun antibody (panel C, lanes 1–6). All these bands were analyzed densitometrically, and the -fold changes were calculated. The data shown here represent three experiments exhibiting similar effects.

activation is not affected by kinase negative MEKK-1 (lane 3). Half of the immunoprecipitated samples were immunoblotted with anti-ERK1/2 antibody (lower panel C, lanes 1–4). These results suggested that MEKK1 negatively regulates OPN-induced ERK activation.

JNK1 Plays a Crucial Role in OPN-induced MEKK1-dependent ERK1/2 Inactivation—Previous results indicated that JNK^{-/-} mice showed enhanced phosphorylation of ERK leading to tumor growth (40); therefore, we have speculated that activation of JNK1 by OPN may play a role in suppression of ERK1/2 activation. Accordingly, cells transfected with wild type MEKK1 were cotransfected with either wild type or dominant negative JNK1 and then treated with OPN. In separate experiments cells transfected with wild type MEKK1 were treated with JNK1 inhibitor, SP600125, and then treated with OPN. Overexpression of wild type MEKK1 alone or with wild type JNK1 suppressed the OPN-induced ERK activation (upper panel D, lanes 1–4), whereas dominant negative JNK1 or SP600125 along with wild type MEKK1 reversed this effect (lanes 5 and 6). Half of the immunoprecipitated samples were immunoblotted with anti-ERK1/2 antibody (lower panel D, lanes 1–6). These data suggested that JNK1 acts as negative regulator in OPN-induced MEKK1-dependent ERK1/2 activation.

To examine whether NIK plays any role in regulation of OPN-induced MEKK-dependent JNK-mediated ERK1/2 inactivation, cells were transfected with wild type NIK and then cotransfected with either wild type or kinase negative MEKK1 and then treated with OPN. The NIK kinase activity was measured by a coupled kinase assay using MEK and ERK as substrates. The data indicated that expression of active or mutant MEKK-1 had no effect on OPN-induced NIK activity (upper panel E, lanes 1–5), suggesting that overexpressed NIK even in the presence of MEKK up-regulates OPN-induced ERK activation. Half of the immunoprecipitated samples were immunoblotted with anti-NIK antibody (middle panel E, lanes 1–5). The level of NIK activity was also detected by using IKK as substrate (lower panel E, lanes 1–5).

OPN Induces $\alpha\beta$ Integrin-mediated NIK and MEKK1-dependent c-Jun Expression—Earlier reports have demonstrated that MEKK in the presence of stimulus induces JNK-dependent c-Jun phosphorylation and enhances AP-1 activation (41).

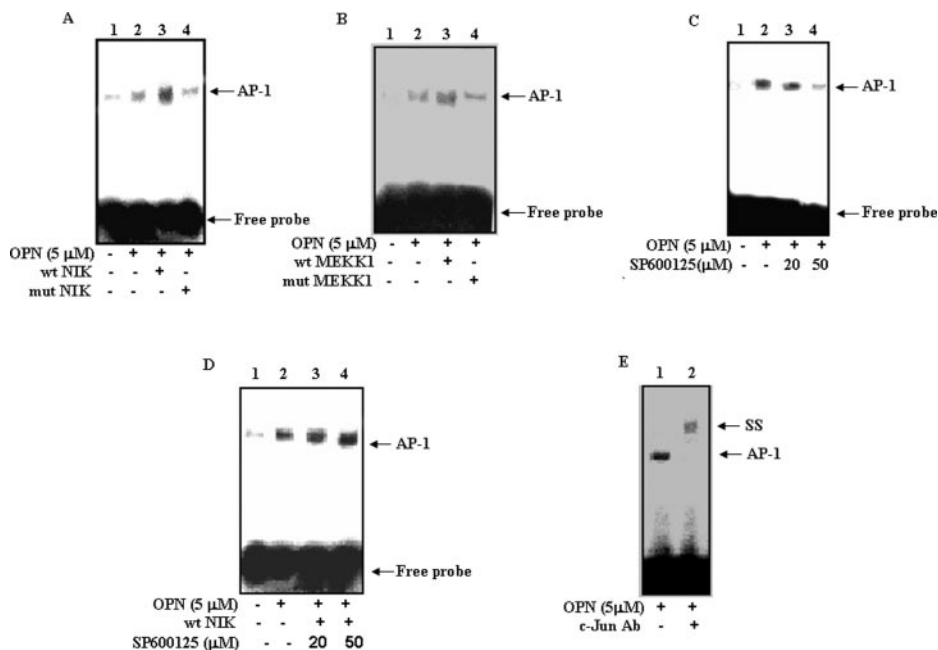
Therefore, we sought to determine whether OPN induces c-Jun expression and whether MEKK1/NIK is involved in this process. Accordingly, cells were treated with 5 μM OPN for 0–4 h. The nuclear extracts were prepared, and the level of c-Jun expression was detected by Western blot analysis using anti-c-Jun antibody. The results indicated that OPN induces c-Jun expression, and maximum expression was observed at 1 h (Fig. 3, panel A, lanes 1–5).

To further confirm that this OPN-induced c-Jun expression occurs through $\alpha\beta$ integrin-mediated pathway, cells were pretreated with anti- $\alpha\beta$ integrin antibody, RGD/RGE peptide, and then treated with OPN for 1 h. The data revealed that $\alpha\beta$ integrin antibody and RGD but not RGE suppressed OPN-induced c-Jun expression (panel B, lanes 1–5).

To examine further whether NIK and MEKK1 play important roles in OPN-induced c-Jun expression, cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 and then treated with OPN for 1 h. The nuclear extracts were prepared, and the level of c-Jun expression was detected by Western blot using anti-c-Jun antibody. Wild type NIK enhanced and kinase negative NIK suppressed OPN-induced c-Jun expression (panel C, lanes 1–4). Similarly, kinase negative MEKK1 inhibited and wild type MEKK1 induced the OPN-induced c-Jun expression (lanes 5 and 6). Moreover, overexpression of wild type NIK, which does not affect OPN-induced JNK phosphorylation and kinase activity (Fig. 1, panel E and Fig. 2, panel B) significantly up-regulate c-Jun expression. These data indicated that OPN induces c-Jun expression through both NIK- and MEKK1-dependent pathways; however, NIK-mediated c-Jun expression occurs in a JNK1-independent manner.

NIK and MEKK1 Play Important Roles in OPN-induced AP-1-DNA Binding—We have reported earlier that OPN induces AP-1-mediated secretion of uPA through c-Src-dependent transactivation of epidermal growth factor receptor in breast cancer cells (15). Therefore, in this paper we have first examined whether NIK and MEKK1 regulate OPN-induced AP-1-DNA binding in B16F10 cells. Accordingly, cells were either treated with 5 μM OPN or transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 and then treated with OPN. The nuclear extracts were prepared and used for EMSA using ³²P-labeled AP-1 oligonucleotides. Wild type NIK

FIG. 4. Panels A and B, OPN induces NIK (panel A)- and MEKK1 (panel B)-dependent AP-1-DNA binding. Cells were either treated with OPN or transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 and then treated with OPN. The nuclear extracts were prepared and analyzed by EMSA (panels A and B, lanes 1–4). Panel C, JNK is involved in OPN-induced AP-1-DNA binding. Cells were pretreated with 0–50 μM SP600125 (JNK inhibitor) and then treated with OPN. The nuclear extracts were analyzed by EMSA (lanes 1–4). Panel D, OPN-induced NIK-mediated AP-1-DNA binding is independent of JNK. Cells were either treated with OPN or transfected with wild type NIK, treated with 0–50 μM SP600125 (JNK inhibitor), and then treated with OPN. The nuclear extracts were analyzed by EMSA (lanes 1–4). Panel E, supershift (SS) assay. The nuclear extracts from OPN treated cells were incubated with anti-c-Jun antibody (Ab) and analyzed by EMSA (lanes 1 and 2). The results shown here represent three experiments exhibiting similar effects.



enhanced and kinase negative NIK suppressed OPN-induced AP-1-DNA binding (Fig. 4, panel A, lanes 1–4). Similarly, wild type MEKK1 induced and kinase negative MEKK1 inhibited OPN-enhanced AP-1-DNA binding (panel B, lanes 1–4). These data suggested that OPN induces AP-1-DNA binding through both NIK- and MEKK1-mediated pathways.

To examine the role of JNK1 on OPN-induced-AP-1-DNA binding, cells were pretreated with 0–50 μM SP600125 (JNK1 inhibitor) and then treated with OPN. The nuclear extracts were prepared and used for EMSA. SP600125 suppressed OPN-induced AP-1-DNA binding in a dose-dependent manner (panel C, lanes 1–4). To ascertain whether OPN-induced JNK1 mediated AP-1-DNA binding is NIK-dependent, cells were transfected with wild type NIK followed by treatment with SP600125 and then stimulated with OPN. The OPN-enhanced AP-1-DNA binding caused by overexpression of wild type NIK was unaltered by SP600125, suggesting that OPN-induced NIK-mediated AP-1-DNA binding is JNK-independent (panel D, lanes 1–4). Whether the band obtained by EMSA is indeed AP-1, the nuclear extracts were incubated with anti-c-Jun antibody and then analyzed by EMSA. The results showed the shift of the AP-1-specific band to a higher molecular weight when the nuclear extracts were treated with anti-c-Jun antibody (panel E, lanes 1 and 2).

OPN Induces NIK- and MEKK1-regulated JNK1-mediated AP-1 Transactivation—To further investigate whether NIK and MEKK1 regulate OPN-induced JNK1-mediated AP-1 transactivation, luciferase reporter gene assay was performed. Cells were transiently transfected with AP-1 luciferase reporter construct (pAP-1-Luc) and then treated with OPN. In separate experiments, cells were individually transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 or wild type and dominant negative JNK1 along with pAP-1-Luc and then treated with OPN. In separate experiments, wild type NIK-transfected cells were cotransfected with pAP-1-Luc, treated with SP600125, and then treated with OPN. The transfection efficiency was normalized by cotransfecting the cells with pRL vector. Changes in luciferase activity with respect to control were calculated. The -fold changes were calculated, and the results are expressed as the means \pm S.E. of three determinations. The values were also analyzed by Student's *t* test ($p < 0.002$). The data showed that

wild type NIK enhanced but kinase negative NIK suppressed OPN-induced AP-1 activity in these cells (Fig. 5, panel A). Similarly, wild type MEKK1 enhanced and kinase negative MEKK1 inhibited OPN-induced AP-1 activity (panel B). Wild type JNK enhanced, whereas dn JNK1 moderately suppressed OPN-induced AP-1 activity (panel C). The enhanced AP-1 transactivation caused by overexpression of wild type NIK followed by OPN treatment was unaffected upon treatment with JNK1-specific inhibitor, SP600125 (panel C). These data indicated that OPN induces AP-1 transactivation through NIK- and MEKK1/JNK-mediated pathways and further suggested that OPN induces a shift in balance toward activation of ERK followed by AP-1 activation.

OPN Stimulates NIK- and MEKK1-mediated c-Jun-dependent uPA Secretion and uPA-dependent MMP-9 Activation—We have recently demonstrated that NIK plays a crucial role in OPN-induced uPA secretion and uPA-dependent MMP-9 activation in B16F10 cells (16). Therefore, we have examined whether MEKK1, JNK1, and c-Jun are involved in OPN-induced uPA secretion. Accordingly, cells were transfected with wild type and kinase negative MEKK1 or wild type and dn JNK1 or wild type and dn c-Jun and then treated with OPN. In separate experiments cells were pretreated with SP600125 (50 μM) and then stimulated with OPN. The cell lysates were analyzed by Western blot using rabbit polyclonal anti-uPA antibody. The data showed that OPN-induced uPA secretion was enhanced when cells were transfected with wt MEKK1 and wt c-Jun and suppressed when transfected with kinase negative MEKK1 and dn c-Jun (Fig. 6, upper panel A, lanes 1–6). Wild type JNK1 stimulated and dn JNK1 or JNK1 inhibitor (SP600125) moderately reduced OPN-induced uPA secretion due to up-regulation of ERK-mediated c-Jun expression leading to activation of AP-1 (upper panel B, lanes 1–5). All these blots were reprobbed with anti-actin antibody (lower panels A and B). All bands were quantified by densitometric analysis, and the -fold changes are calculated (panels A and B). These data further demonstrated that OPN induces uPA secretion through both NIK/ERK as well as MEKK1/JNK-mediated pathways.

To examine whether OPN-induced NIK/MEKK1-mediated uPA secretion leads to MMP-9 activation, cells were transfected with wild type and kinase negative MEKK1 or wild type

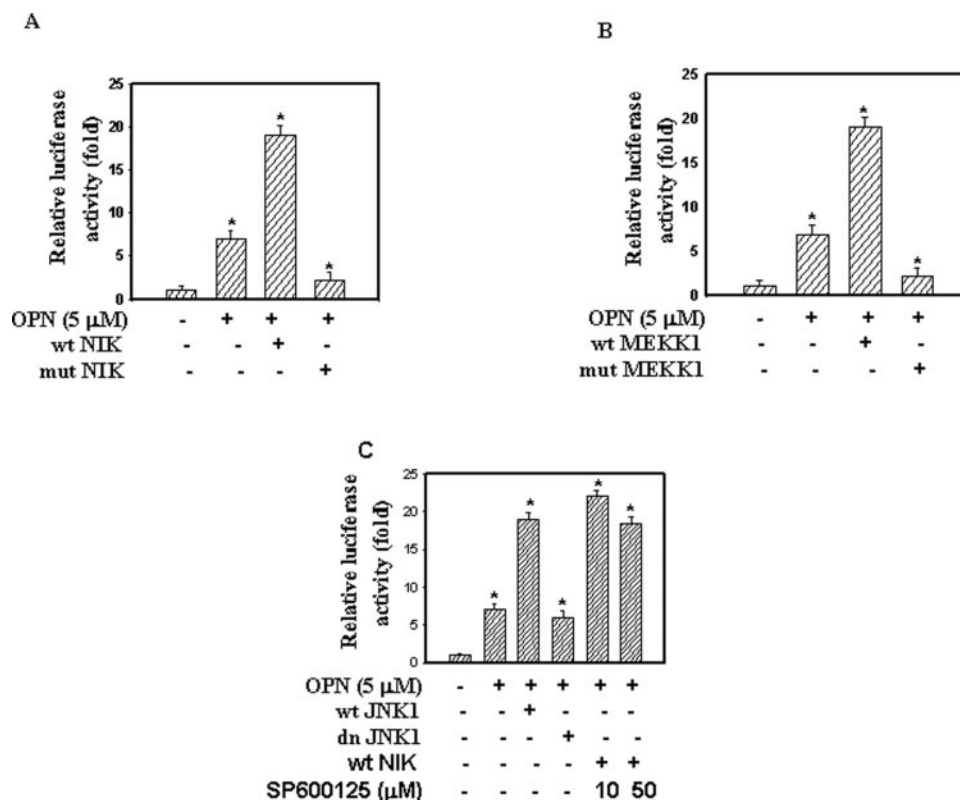


FIG. 5. Panels A and B, OPN enhances NIK (panel A)- and MEKK1 (panel B)-dependent AP-1 transactivation. Cells were transiently transfected with luciferase reporter construct (pAP-1-Luc). In separate experiments cells were individually transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 along with pAP-1-Luc. The transfected cells were treated with 5 μ M OPN. Cell lysates were used to measure the luciferase activity (panels A and B). The values were normalized to Renilla luciferase activity. Panel C, JNK is differentially regulated in OPN-induced NIK-dependent AP-1 transactivation. Cells were transfected with wild type and dominant negative JNK1 along with pAP-1-Luc and treated with 5 μ M OPN. In other experiments cells were transfected with wild type NIK along with pAP-1-Luc and treated with 0–50 μ M SP600125 and then with OPN. Cell lysates were used to measure the luciferase activity (panel C). The -fold changes were calculated, and means \pm S.E. of triplicate determinations are plotted. The values were also analyzed by Student's *t* test (*, $p < 0.002$).

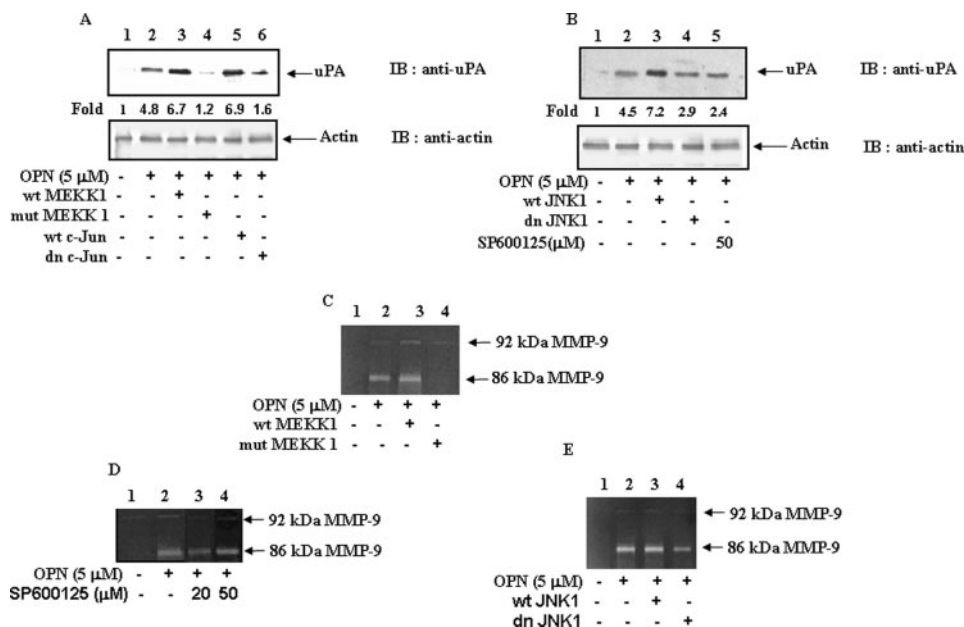


FIG. 6. Panel A, OPN stimulates MEKK1- and c-Jun-mediated uPA secretion. Cells were either treated with OPN or transfected with wild type and kinase negative MEKK1 or wild type and dn c-Jun and then treated with OPN. The levels of uPA in the cell lysates were analyzed by Western blot (IB) using anti-uPA antibody (upper panel A, lanes 1–6). The same blots were reprobed with anti-actin antibody (lower panel A, lanes 1–6). Panel B, JNK plays a crucial role in OPN-induced uPA secretion. Cells were transfected with wild type and dn JNK1 or pretreated with 50 μ M of SP600125 (JNK inhibitor) and then treated with OPN. The level of uPA in the cell lysates was analyzed by Western blot using anti-uPA antibody (upper panel B, lanes 1–5). The same blots were reprobed with anti-actin antibody (lower panel B, lanes 1–5) as the loading control. All these bands were quantified densitometrically. Panels C–E, JNK is differentially regulated in OPN-induced MEKK1-dependent pro-MMP-9 activation. Cells were individually transfected with wild type and kinase negative MEKK1 or wild type and dn JNK1 or pretreated with SP600125 and then treated with OPN. The conditioned media were collected, and the activity of MMP-9 was examined by gelatin zymography (panels C–E, lanes 1–4). The data shown here represent three experiments exhibiting similar effects.

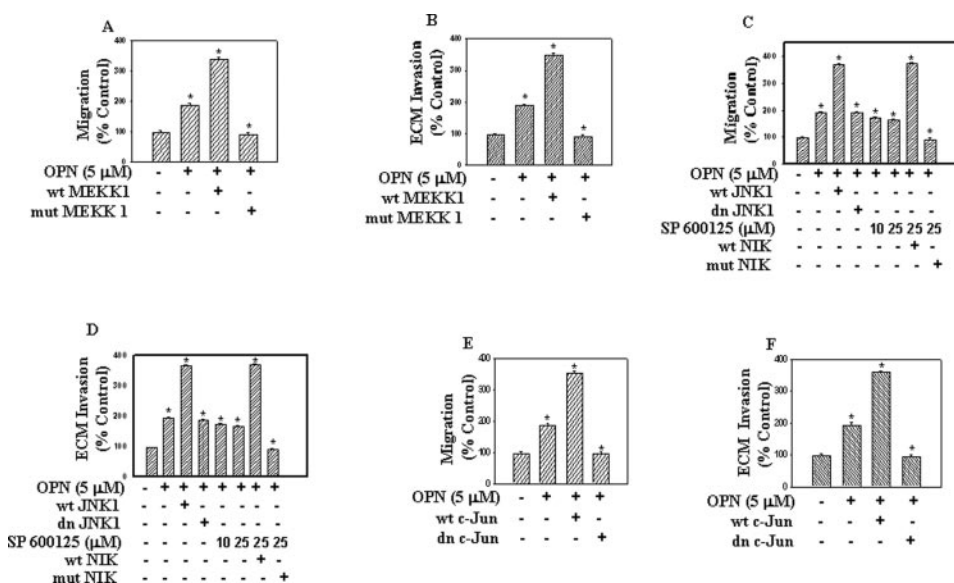


FIG. 7. Roles of MEKK1, JNK1, and c-Jun in OPN-stimulated cell migration and chemoinvasion. The migration assay was conducted either by using untreated cells or cells transfected with wild type and kinase negative MEKK1 or wild type and dn JNK1 or wild type and dn c-Jun. The purified human OPN (5 μ M) was added in the upper chamber. The treated or transfected cells were used for migration assay. Note that OPN-induced migration was suppressed by kinase negative MEKK1 and dn c-Jun and enhanced by wt MEKK1, wt JNK1, and wt c-Jun. In separate experiments cells were treated with 0–25 μ M SP600125 or transfected with wild type or kinase negative NIK and then treated with 25 μ M SP600125. These transfected or treated cells were used for migration assay (panels A, C, and E). dn JNK-1 and SP600125 did not alter OPN-induced cell migration (panel C). Note that JNK1 plays a differential role in OPN-induced NIK/MEKK1-dependent cell migration. The same results were obtained in chemoinvasion assays (panels B, D, and F). The results are expressed as the means \pm S.E. of three determinations.

and dn JNK1 or pretreated with SP600125 and then treated with OPN. The conditioned media were collected and used for gelatinolytic activity by zymography. Increased levels of MMP-9 activation (86 kDa) were observed when cells were treated with OPN (panels C–E, lane 2). Almost no MMP-9-specific band was detected in the untreated cells (lane 1). Wild type MEKK1 enhanced, whereas kinase negative MEKK1 suppressed OPN-induced MMP-9 activation (panel C, lanes 1–4). Wild type JNK1 enhanced, and dn JNK1 moderately reduced OPN-induced MMP-9 activation (panel E, lanes 1–4). JNK1 inhibitor SP600125 retained OPN-induced MMP-9 activation due to up-regulation of ERK activation (panel D, lanes 1–4). These data suggested that OPN induces uPA expression and uPA-dependent MMP-9 activation through both NIK/ERK1/2- and MEKK1/JNK1-mediated pathways and further demonstrated that OPN induces a shift in balance toward activation of ERK, thereby retaining the uPA secretion and MMP-9 activation.

MEKK1, JNK1, and c-Jun Play Crucial Roles in OPN-induced α v β 3 Integrin-mediated Cell Migration and Chemoinvasion—We have shown that OPN induces α v β 3 integrin-mediated NIK/ERK and MEKK1/JNK1-dependent c-Jun expression leading to uPA secretion and uPA-dependent MMP-9 activation. Therefore, we have examined whether these OPN-induced NIK/MEKK1-dependent MMP-9 activations play any role in cell migration and chemoinvasion. Accordingly, cells were either transfected with wild type and kinase negative MEKK1 or wild type and dn JNK1 or wild type and dn c-Jun in the presence of Lipofectamine Plus and then used for migration or chemoinvasion assay. In separate experiments cells were pretreated with JNK1 inhibitor (SP600125) or transfected with wild type and kinase negative NIK and then treated with JNK1 inhibitor. OPN was used in the upper chamber. The data showed that wild type MEKK1, JNK1, and c-Jun enhanced and mutant MEKK1 and c-Jun suppressed OPN-induced cell migration (Fig. 7, panels A, C, and E) and chemoinvasion (panels B, D, and F). The data also indicated that dn JNK1 and SP600125 unaltered the OPN-induced cell migration (panel C)

TABLE I
NIK and MEKK1 enhances OPN-induced tumor growth in nude mice

B16F10 cells were treated with 10 μ M OPN for 16 h and injected into nude mice (NMRI). The injection was performed twice a week for 4 weeks. In separate experiments cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 followed by treatment with OPN and then injected into nude mice. The mice were killed, and the tumor weights were measured and analyzed statistically by Student's *t* test ($p < 0.002$). Mice injected with cells in phosphate-buffered saline were used as controls.

No. nude mice	Transfection/Treatment	Tumor weight (-fold changes)
4	Control (PBS)	1.0 \pm 0.17
4	OPN (10 μ M)	3.1 \pm 0.15
4	Wt NIK + OPN (10 μ M)	6.1 \pm 0.14
4	Mut NIK + OPN (10 μ M)	1.4 \pm 0.16
4	Wt MEKK1 + OPN (10 μ M)	5.4 \pm 0.12
4	Mut MEKK1 + OPN (10 μ M)	1.2 \pm 0.15

and chemoinvasion (panel D). The enhanced migration and invasion caused by overexpression of wild type NIK is unaffected by cells treated with JNK1 inhibitor (panels C and D). However, cells transfected with mutant NIK followed by treatment with JNK1 inhibitor suppressed OPN-induced migration and invasion (panel C and D), suggesting that NIK-regulated migration and invasion are independent of JNK, and both the pathways synergistically contribute the OPN-induced cell migration and chemoinvasion. These data demonstrated that OPN-induced uPA secretion and uPA-dependent pro-MMP-9 activation are regulated by NIK/ERK and MEKK1/JNK1 pathways, and all of these ultimately control the motility and invasiveness of B16F10 cells.

OPN Induces NIK/MEKK1-dependent c-Jun Expression, AP-1-DNA Binding, uPA Secretion, and MMP-9 Activation in Tumor of Nude Mice—The *in vitro* data prompted us to examine whether NIK and MEKK1 play any role in OPN-induced c-Jun expression, AP-1-DNA binding, uPA secretion, and MMP-9 activation in the tumors of nude mice. Accordingly, cells were either treated with OPN or transfected with wild type and

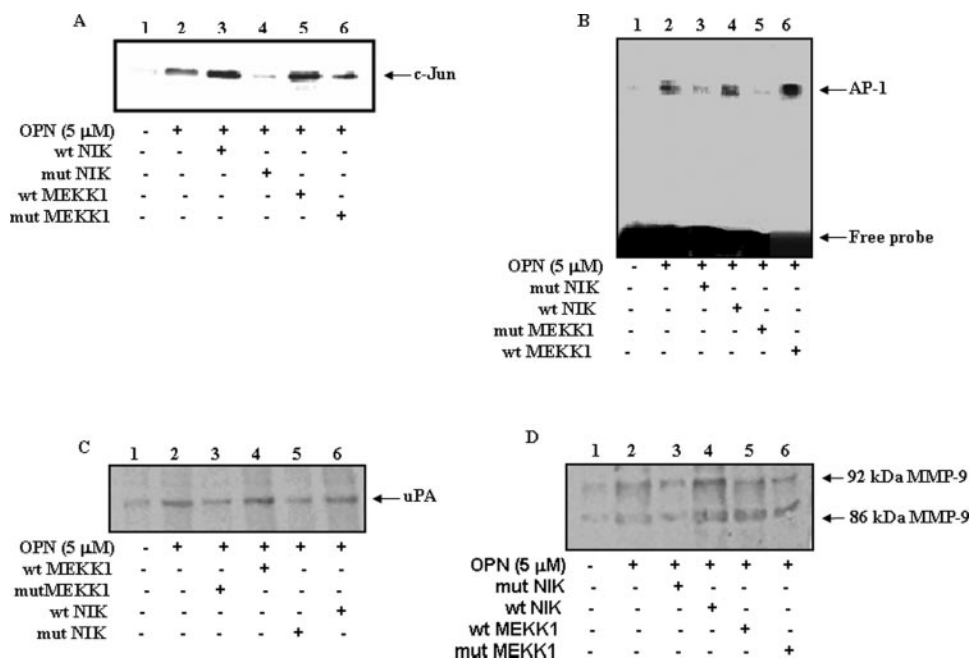


FIG. 8. Panels A and B, OPN enhances NIK/MEKK-dependent c-Jun expression and AP-1-DNA binding in tumors of nude mice. The samples obtained from tumors generated by wild type and kinase negative NIK or wild type and kinase negative MEKK1 were lysed, and nuclear extracts were prepared and subjected to Western blot using anti-c-Jun antibody (panel A, lanes 1–6). The same nuclear extracts were used for EMSA assays (panel B, lanes 1–6). Panels C and D, OPN induces NIK/MEKK-dependent uPA expression and MMP-9 activation in same tumors of nude mice. The samples obtained from tumors generated by wild type and kinase negative NIK or MEKK1 were lysed, and the levels of uPA were analyzed by Western blot using anti-uPA antibody (panel C, lanes 1–6). The level of MMP-9 in these samples was also analyzed by Western blot using anti-MMP-9 antibody (panel D, lanes 1–6). The data shown here represent three experiments exhibiting similar effects.

kinase negative NIK or wild type and kinase negative MEKK1 followed by treatment with OPN and then injected subcutaneously into the flanks of nude mice. Table I shows the -fold change of tumor weight grown in 4-week-old nude mice. There were at least 6- and 5.4-fold increased in tumor weight when wild type NIK or wild type MEKK1-transfected cells were injected, respectively. Four mice were used in each set of experiments. The changes in tumor weights were analyzed statistically by Student's *t* test ($p < 0.002$). The tumor samples were lysed, and the level of c-Jun expression in the nuclear extract was detected by Western blot using anti-c-Jun antibody. The AP-1-DNA binding in the nuclear extract was performed by EMSA. Both wild type NIK and wild type MEKK1 showed significantly higher levels of c-Jun expression (Fig. 8, panel A, lanes 1–6) and AP-1-DNA binding (panel B, lanes 1–6) compared with cells treated with OPN alone or transfected with kinase negative NIK (mut NIK) or mut MEKK1.

To further examine the levels of uPA and MMP-9 in these tumors, the samples were lysed, and the levels of uPA and MMP-9 were analyzed by Western blot using anti-uPA and anti-MMP-9 antibody, respectively. The results indicated that tumor generated by injecting the mice with wild type NIK and MEKK1-transfected cells showed higher level of uPA expression (panel C, lanes 1–6) and MMP-9 activation (panel D, lanes 1–6) compared with cell treated with OPN alone or transfected with mutant NIK or MEKK1. These data demonstrated that OPN induces both NIK- and MEKK1-mediated AP-1 activation leading to uPA secretion and pro-MMP-9 activation through JNK1-dependent/independent pathways in tumor of nude mice and these data corroborates with *in vitro* data.

DISCUSSION

In a recent study (16) we have demonstrated that OPN stimulates NIK-dependent NF κ B-mediated uPA secretion and uPA-dependent pro-MMP-9 activation that controls cell motility and tumor growth through both IKK and ERK1/2-mediated pathways in murine melanoma cells. In this paper we have

delineated the molecular mechanism by which OPN regulates NIK/MEKK1-dependent c-Jun expression and AP-1 transactivation and the differential role of JNK1 in these activation processes in murine melanoma cells. We have shown that OPN induces α v β 3 integrin-mediated MEKK1 phosphorylation leading to c-Jun activation in a JNK-dependent manner. The data also revealed that OPN induces NIK activation, which further enhances c-Jun expression, leading to AP-1 transactivation in a JNK-independent pathway. Overexpression of MEKK1 leads to sustained activation of JNK, resulting in a negative cross-talk between MEKK1/JNK and NIK/ERK pathways. OPN binding to α v β 3 integrin induced NIK/MEKK1-dependent c-Jun expression, which ultimately stimulates uPA secretion and uPA-dependent pro-MMP-9 activation that enhances cell migration, chemoinvasion, and tumor growth.

OPN plays a significant role in tissue remodeling processes such as bone resorption, angiogenesis, wound healing, and tissue injury as well as certain diseases such as restenosis, atherosclerosis, tumorigenesis, and autoimmune diseases (6–8). Integrins are cell surface glycoproteins that bind to the extracellular matrix proteins. Recent studies have demonstrated that down-regulation of NIK activation does not affect tumor necrosis factor α -induced JNK activation (42). It has been also reported that ERK and JNK pathways play crucial roles in regulating MMP-9 activation and cell motility in growth factor-stimulated human epidermal keratinocytes (43). These results prompted us to investigate whether binding of OPN to α v β 3 integrin receptors regulates JNK1 activation and whether NIK is involved in this activation process. In this study we have demonstrated that OPN induces α v β 3 integrin-mediated MEKK1 and JNK1 phosphorylations in B16F10 cells. Pretreatment of cells with anti- α v β 3 integrin antibody and RGD but not RGE peptide inhibited OPN-induced MEKK1 and JNK1 phosphorylations, indicating that α v β 3 is involved in this process. Furthermore, OPN-induced JNK1 activation is MEKK1-dependent but NIK-independent. This was confirmed

by the fact that transient overexpression of wild type MEKK1 enhanced and kinase negative MEKK1 suppressed OPN-induced JNK1 phosphorylation and kinase activity, whereas overexpression of wild type and kinase negative NIK does not affect OPN-induced JNK1 activation.

MEKK1, a Ser/Thr protein kinase has been reported as a mitogen-activated protein kinase kinase kinase that activates JNK via phosphorylation of its downstream kinase mitogen-activated protein kinase kinase 4 (18). Shen *et al.* (44) have recently reported that sustained activation of JNK blocks ERK activation in response to mitogenic factors like epidermal growth factor and phorbol 12-myristate 13-acetate. Growing evidence also indicated that cross-regulation between JNK and ERK may play an important role in determining cell survival or death. These results prompted us to examine whether overexpression of MEKK1 leads to enhanced JNK activation and whether this activation may affect OPN-induced NIK-mediated ERK activation. Our data demonstrated a negative cross-talk between OPN-induced NIK/ERK and MEKK1/JNK activation and further suggested that sustained activation of JNK resulted in the attenuation of ERK activation. Previous studies have indicated that MEKK1 also has the ability to activate ERK, but the effect is less potent (19). This may be implicated to the short and long phase of MEKK1 activation, which results in a different cellular response; that is, a short phase activation that leads to ERK activation and a long phase activation that results in inhibition of ERK activation. Also, the inhibition of OPN-induced NIK-mediated ERK activation caused by overexpression of wild type MEKK1 involves the ability of MEKK1 to activate the JNK pathway. These implications delineate a mechanism in which treatment with the same agonist may result in a different cellular outcome depending on the duration of treatment. These data are consistent with the recent report that JNK1 deficiency stimulates 12-*O*-tetradecanoylphorbol-13-acetate-induced ERK phosphorylation, leading to enhanced skin tumorigenesis (40).

It is well established that JNK, a member of the mitogen-activated protein kinase family, could be phosphorylated after exposure to ultraviolet irradiation, growth factors, or cytokines, which in turn phosphorylates specific serine residues (serine 63 and serine 73) of c-Jun and enhances the AP-1 transcriptional activity. AP-1, a family of transcription factors, consists of homo or heterodimers of Jun, Fos, or activating transcription factor protein (44–46). Previous reports have demonstrated that AP-1 is involved in several cellular processes such as cell growth, apoptosis, and cell motility (45). In addition, AP-1 activity is elevated in a number of pathological conditions. Natoli *et al.* (42) have reported that overexpression of NIK, which does not activate JNK, strongly activates transcription directed by a canonical AP-1 site. Because we have shown that OPN induces MEKK1-dependent but NIK-independent JNK phosphorylation and AP-1 response element is present in the promoter region of MMP-9 gene, we sought to determine the level of c-Jun expression upon OPN stimulation. OPN enhances the expression of c-Jun, resulting in enhancement of AP-1-DNA binding activity. Our data also indicated that OPN induces both NIK- and MEKK1-mediated c-Jun expression, leading to AP-1-DNA binding and AP-1 transactivation. The enhanced AP-1-DNA binding caused by overexpression of wild type NIK was unaffected upon inhibition of JNK activation by SP600125, a specific JNK inhibitor. These data suggested that overexpression of NIK, which does not affect JNK activation significantly, up-regulates AP-1-DNA binding and transcriptional activity, indicating that OPN induces NIK-dependent AP-1 activation, which is independent of JNK.

Signals transduced by cell adhesion molecules play an im-

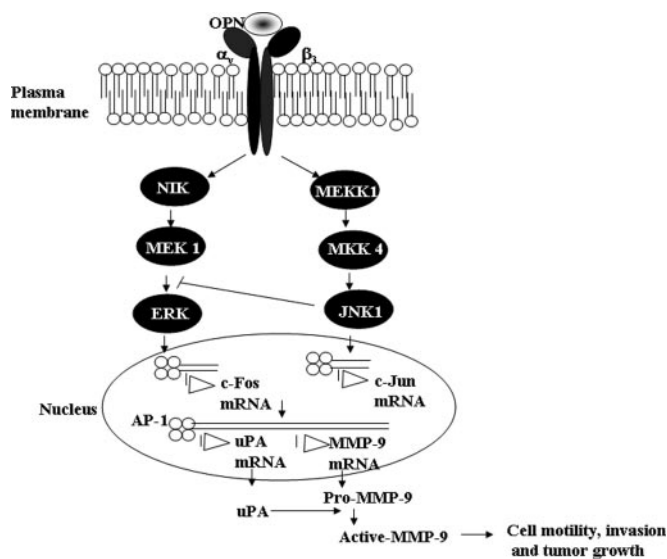


FIG. 9. Molecular mechanism of OPN-induced NIK/MEKK1-dependent AP-1 activation, AP-1-mediated uPA secretion, and MMP-9 activation through differential activation of JNK1. Binding of OPN to $\alpha 5 \beta 3$ integrin induced the phosphorylation and activation of MEKK1 which induces c-Jun-mediated AP-1 activation in a JNK1-dependent manner. In addition, OPN also induced NIK-dependent c-Jun-mediated AP-1 activation through JNK1-independent pathway. These lead to a cross-talk between NIK/ERK and MEKK1/JNK1 pathways. Both NIK and MEKK1 in the presence of OPN regulate AP-1-dependent uPA secretion and MMP-9 activation, and all of these control the cell motility, invasion, and tumor growth.

portant role in tumor cell attachment, motility, and invasion, all of which regulate metastasis. OPN, an ECM protein, plays a significant role in cell adhesion, migration, and metastasis. MMPs are a family of Zn^{2+} -dependent endopeptidases that are responsible for remodeling of the extracellular matrix and degradation of ECM proteins. MMP-9 is known to degrade basement membrane, which normally separates the epithelial from stromal compartment. Elevated levels of MMP-9 have been reported in various cancers. Several studies have shown a correlation between MMP-9 expression and the metastatic potential of tumor (47). Kim *et al.* (48) also demonstrated that MMP-9 activity but not MMP-2 activity significantly affects tumor intravasation into blood vessel, and uPA is required for pro-MMP-9 activation (49). Several other reports have indicated the correlation between uPA expression and metastatic potential and shown that uPA plays major role in regulating MMPs activation (16, 50). In this study we have reported that OPN induces uPA secretion and uPA-dependent pro-MMP-9 activation through NIK/ERK- and MEKK1/JNK-mediated AP-1-dependent pathways. Overexpression of wild type MEKK1 and c-Jun enhanced, and kinase negative MEKK1 and dn c-Jun suppressed the OPN-induced uPA secretion, demonstrating that OPN regulates uPA secretion through MEKK1/c-Jun-mediated pathways. Similarly, overexpression of wild type JNK1 enhanced OPN-induced uPA secretion and MMP-9 activation. Moreover, cells transfected with wild type NIK followed by treatment with JNK1 inhibitor enhanced, whereas cells transfected with kinase negative NIK followed by treatment with JNK1 inhibitor suppressed the OPN-induced cell migration and invasion, indicating that OPN regulates these effects through both NIK- and JNK-mediated pathways. Wild type MEKK1, JNK1, and c-Jun enhanced, and kinase negative MEKK1 and dn c-Jun suppressed OPN-induced cell migration and ECM invasion. However, transfection of cells with the dominant negative form of JNK1 or treatment with SP600125 moderately inhibits OPN-induced uPA secretion or uPA-dependent pro-MMP-9 activation, cell migration, and ECM inva-

sion. This may be due to the cross-talk between MEKK1/JNK and NIK/ERK pathways. These data are consistent with the recent data reported by Chen *et al.* (40) that disruption of the JNK1 gene resulted in an increase in ERK phosphorylation leading to enhancement of skin tumorigenesis. The *in vitro* data are also supported by *in vivo* data which showed that OPN induced both NIK- and MEKK1-mediated c-Jun expression, leading to uPA-dependent pro-MMP-9 activation in tumors of nude mice. These data demonstrated that OPN induces NIK/MEKK1-regulated AP-1-mediated uPA-dependent pro-MMP-9 activation, cell motility, and tumor growth through differential activation of JNK1 in B16F10 cells.

In summary, we have demonstrated for the first time that OPN induces both NIK- and MEKK1-mediated c-Jun expression, leading to AP-1 transactivation in B16F10 cells. Ligation of OPN to $\alpha\beta 3$ integrin receptor induces the phosphorylation and kinase activity of JNK1. This was blocked by kinase negative MEKK1 but unaffected by kinase negative NIK, indicating that OPN-induced NIK-mediated AP-1 transactivation is JNK-independent. OPN also induces a negative cross-talk between MEKK1/JNK and NIK/ERK pathways. Overexpression of wild type MEKK1 attenuates OPN-induced NIK-mediated ERK activation, which is restored upon JNK inhibition. Taken together, OPN-induced uPA-dependent pro-MMP-9 activation, cell motility, and tumor growth through both NIK- and MEKK1-mediated c-Jun expression and JNK1 plays a differential role in modulating these processes (Fig. 9). These findings may be useful in designing novel therapeutic interventions that block the OPN-regulated NIK- and MEKK1-dependent c-Jun expression and AP-1 transactivation through differential activation of JNK1, resulting in reduction of uPA secretion and MMP-9 activation and consequent blocking of cell motility, invasiveness, and metastatic spread of malignant melanoma.

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