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/IkB kinase-dependent nuclear factor-κB (NFκB)-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 αβ3 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 NIK 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 phosphorylation and activation. Overexpression of wild type but not kinase negative MEKK1 expression of constitutively active forms of MEKK1 leads to JNK activation via phosphorylation of its upstream kinase, nuclear factor-inducing kinase (NIK) is another member of the mitogen-activated protein kinase (MAPK) family that has 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 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 a constitutively active form 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 kinase family that has been implicated in NFκB 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 matrix (ECM) protein family (1, 2). OPN acts both as chemo- kine 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 proliferation, ECM-invasion, and cell proliferation by interacting with its receptor αβ3 integrin in different cell types (6). Previous data indicated that OPN is

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

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‡ The abbreviations used are: OPN, osteopontin; NIK, nuclear factor-inducing kinase; MEKK1, mitogen-activated protein kinase kinase; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; AP-1, activating protein-1; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; Luc, luciferase; ECM, extracellular matrix; uPA, urokinase type plasminogen activator; MMP-9, matrix metalloproteinase-9; IKK, IκB 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 inactive plasminogen plasmin 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 NFkB-mediated pro-MMP-2 activation through IкBa/IKK signaling in murine glioma C6 cells (9). In the present study, we show that OPN induces uPA and c-Jun expression in murine melanoma (B16F10) cells that are 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 a well-defined.

In this paper we have demonstrated that OPN induces αvβ3 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/JNK 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 αvβ3 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-IKKa/β, 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-human αvβ3 integrin antibody was from Chemicon International. Lipo-fection Plus, GRGDSP, and GRGESP were obtained from Invitrogen. Mouse monoclonal anti-phospho-uPA receptor antibody was purchased from BioGenex. 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 μg/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 MEKK1 transfections were constructed (pcDNA3 MEKK1 and pcDNA3 FlagMEKK1 K432M) were kind gifts from Prof. Tom Maniatis (Harvard University, Cambridge). The wild type c-Jun in pGL3B10B and dominant negative c-Jun in pELF1N were gifts from Dr. Jalam (Ochsner Clinic Foundation, New Orleans, LA). The wild type and dominant negative forms of MEKK1 c-Jun 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 1% fetal calf serum. These cells were transiently transfected with reporter vector, reporter vector containing dominant negative c-Jun, and 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 were detected by ECL detection system (Amersham Biosciences). As loading controls, the expression of actin was also detected by reprobing the blots with rabbit anti-actin antibody.

Immunoprecipitation—To examine the effect of OPN in regulation of MEKK1 phosphorylation, cells were treated with 5 μM OPN at 37 °C for 40–60 min. In separate experiments, cells were pretreated with αvβ3 integrin antibody (20 μg/ml), GRGDSP or GRGESP peptide (10 μM) and then treated with 5 μM 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-phospho-serine antibody. As loading control, same blots were reprobed with rabbit polyclonal anti-MEKK1 antibody.

To delineate the role of OPN in regulation of JNK-1 phosphorylation, cells were treated with 5 μM OPN for 0–90 min at 37 °C. In separate experiments cells were pretreated with anti-αvβ3 integrin antibody (20 μg/ml), GRGDSP or GRGESP peptide (10 μM) and then treated with 5 μM 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 reprobed 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 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 μM SP600125 (JNK-1 inhibitor) and then treated with 5 μM OPN for 24 h. To examine whether c-Jun plays any role in OPN-induced uPA secretion, the 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 were detected by ECL detection system (Amersham Biosciences). As loading controls, the expression of actin was also detected by reprobing the blots with rabbit anti-actin antibody.
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and then treated with 5 μM OPN for 15 min. Cell lysates were immu
noprecipitated with rabbit polyclonal anti-JNK-1 antibody and ana
lyzed by Western blot using anti-phosphotyrosine antibody. The same
blots were reprobed with anti-JNK-1 antibody.

**Nuclear Extracts and Western Blot**—To check the level of c-Jun expres
sion in the nucleus, cells were treated with 5 μM OPN for 0–4 h at 37 °C.
In separate experiments, cells were pretreated with anti-αvβ3 integrin antibody (20 μg/ml), GRGDSP or GRGESP peptide (10 μM) or transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 and then treated with 5 μM 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 μM 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 × g for 5 min at 4 °C. The nuclear pellet was extracted in nuclear extraction buffer (20 mM Hepes (pH 7.9), 0.4 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM dithi
othing to) and centrifuged at 12,000 × 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-
duced AP-1 DNA binding, 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 for 1 h at 37 °C. The nuclear extracts were prepared as described earlier (16). To inves
tigate 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 SP600125. Transfected or treated cells were further
nucleated 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 efficien

cy was normalized by cotransfecting the cells with pRL vector and then treated with 5 μM OPN or transfected with wild type and kinase negative MEKK1 or wild type and kinase negative MEKK1 and then treated with OPN. The nuclear extracts (10 μg) were incubated with 16 fmol of 32P-labeled double-stranded AP-1 oligonucleotide (5′-CGG TTG ATG ACT CAG CCG GAA-3′) in binding buffer (25 mM Hepes (pH 7.9), 10 mM NaF, 10 mM p-nitrophosphoryl phosphate, 300 μM Na3VO4, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM DTT, and 0.25% Nonidet P-40). The supernatant was obtained by centrifugation at 12,000 × g for 10 min at 4 °C. The cell lysates (300 μg) containing equal amount of total protein 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-nitrophosphoryl phosphate, 300 μM Na3VO4, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT) containing 10 μM ATP and 3 μCi 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 resulting half of the immu
noprecipitated samples were subjected to SDS-PAGE and analyzed by Western blot using anti-JNK-1 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 NIK-1 or treated with 20 μM SP600125 (JNK1 inhibitor) and then treated with OPN. The cells lysates containing equal amount of total proteins were immunoprecipitated with anti-
ERK1/2 antibody. The half of the immunocomplexes were incubated with 2 μg of myelin basic protein in kinase assay buffer supplemented with 10 μM ATP and 3 μCi 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 as-
sayed as described previously (39). Briefly, cells were treated with 5 μM OPN for 15 h and then transfected with wild type and kinase negative MEKK1 and then treated with 5 μM OPN. The conditioned medium was collected, and the samples contain

ing 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 mM ZnCl2, 1% (v/v) Triton-X100, and 0.02% (w/v) Na3VO4) for 16 h. The gels were stained with Coomassie Blue and destained. Neg
ative staining showed the zones of gelatinolytic activity.

**In Vitro Kinase Assay**—To examine the effect of OPN on JNK activ
ity, the semiconfluent cells were treated with 5 μM 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 μM 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

tein 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 anti
body. 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 in
vasion 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 experi
ments 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 × g for 10 min. The cell suspension (5 × 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 μg) 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 on 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 experi
ments were repeated in triplicate. Preimmune IgG served as nonspec
ific control.

**In Vivo Tumorigenicity Experiments**—The tumorigenicity experi
ments were performed as described previously (12, 13, 16). The cells were treated in the absence or presence of purified human OPN (10 μM) 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 μM OPN. After that, the cells (5 × 106/ml) were detached and

*Withdawn*
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**RESULTS**

**OPN Induces αvβ3 Integrin-dependent MEKK1 and JNK1 Phosphorylations**—To investigate the role of OPN on MEKK1 and JNK1 phosphorylations and to demonstrate the involvement of αvβ3 integrin in this activation process, B16F10 cells were treated with 5 μM OPN at 37 °C or pretreated with anti-αvβ3 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-phosphotyrosine antibody. As shown in Fig. 1, upper panels A and C, lanes 1–5. Pretreatment of cells with anti-αvβ3 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-JNK1 antibody (lower panels B and D, lanes 1–5). All these bands were analyzed densitometrically, and the -fold changes were calculated. These results demonstrated that OPN induces MEKK1 and JNK1 phosphorylations through αvβ3 integrin-mediated pathway.

**MEKK1 but Not NIK Is Required for OPN-induced αvβ3 Integrin-mediated JNK1 Phosphorylation**—Because we have reported earlier that OPN induces NIK-dependent NFκB-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. As shown in Fig. 1, upper panel 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-
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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 blotting anti-MEKK1 (upper panel A, lanes 1–4) or anti-NIK antibody (lower panel A, lanes 1–4). Panel C: overexpression of MEKK1 attenuates JNK1 activity. Cells were transfected with wild type and kinase negative MEKK1 or wild type and kinase negative NIK 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 1–5). The JNK1 activity was also assayed under the same conditions using c-Jun as substrate (lower panel C, lanes 1–6). The data shown here represent three experiments exhibiting similar effects.

Fig. 3. Overexpression of 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

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, MEKK1 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 panel 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
activation is not affected by kinase negative MEKK-1 (lane 3). Half of the immunoprecipitated samples were immunoblotted with anti-ERKI/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 ERKI/2 Inactivation—**Previous results indicated that JNK1−/− mice showed enhanced phosphorylation of ERK leading to tumor growth (40); therefore, we have speculated that MEKK in the presence of stimulus induces JNK-dependent ERK1/2 activation. Accordingly, cells 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 (panels A, B, lanes 1–5). All these bands were analyzed densitometrically, and the fold changes were calculated. The data shown here represent three experiments exhibiting similar effects.

Fig. 3. Panels A and B, OPN enhances avß3 integrin-mediated c-Jun expression. Cells were treated with 5 μM OPN for 0–4 h or pretreated with anti-avß3 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.

**WITHDRAWN**
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, NIK 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). 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.

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-enhanced AP-1-DNA binding (panel B, lanes 1–4). These data suggested that OPN induces AP-1-DNA binding through NIK- and MEKK1-mediated pathways.

To examine the role of JNK1 on OPN-induced AP-1-DNA binding, cells were pretreated with SP600125 (50 μM) and then stimulated with OPN. The nuclear extracts were prepared and analyzed by EMSA (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 ± 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). These data indicated that OPN induces AP-1 transactivation caused by overexpression of wild type NIK and MEKK1 and that OPN induces AP-1 transactivation through NIK- and MEKK1-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 and kinase negative NIK and then treated 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 reprobed 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 μM 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 μM OPN. In other experiments cells were transfected with wild type NIK along with pAP-1-Luc and treated with 0–50 μM SP600125 and then with OPN. Cell lysates were used to measure the luciferase activity (panel C). The -fold changes were calculated, and means ± 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 μM 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.

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S.E. of triplicate determinations are plotted. The values were also analyzed by Student’s t test (*, p < 0.002).
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 μg) 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 μM SP600125 or transfected with wild type or kinase negative NIK and then treated with 25 μM SP600125. These transfected or treated cells were used for migration assay (panels A, C, and E). dn JNK1 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 assay (panels B, D, and F). The results were expressed as the means ± S.E. of three determinations.

Tumor weight of Nude Mice—

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

We have shown that OPN induces αvβ3 integrin-mediated cell migration and chemoinvasion.—We have shown that OPN induces αvβ3 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 NIK 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) 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 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 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.

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 NFκB-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 transactiva-

Kinase negative NIK or wild type and kinase negative MEKK1 followed by treatment with OPN and then injected subcutane-

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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 moderate inhibition of ERK activation. These implications delineate a role of MEKK1 in the regulation of OPN-induced NIK-mediated ERK activation. The enhanced AP-1-DNA binding caused by overexpression of MEKK1 leads to enhanced JNK activation and 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 activation 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 important role in cell adhesion, migration, and metastasis. OPN, an ECM protein, plays a significant role in cell adhesion, migration, and metastasis. OPN induces uPA secretion and uPA-dependent pro-MMP-9 activation through differential activation of JNK1. Binding of OPN to αvβ3 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 pathways. These lead to a cross-talk between NIK/ERK and MEKK1/JNK 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 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-
Cross-talk between OPN-induced JNK and ERK Pathways

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