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Matrix metalloproteinase-2: Mechanism and regulation of NF-κB-mediated activation and its role in cell motility and ECM-invasion

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Matrix metalloproteinases belong to a family of enzymes that degrade the extracellular matrix (ECM) components and play an important role in tissue repair, tumor invasion, and metastasis. ECM proteins, cytokines, and certain other factors regulate MMP activity. OPN, an ECM protein, has been found to be overexpressed in various cancers, and it has been shown to correlate with the metastatic potential. Although such reports indicate that OPN plays an important role in the ability of tumor cells to survive and metastasize to secondary sites, the mechanism by which OPN regulates these processes is yet to be understood. In this study we report that native purified human OPN can induce cell migration and ECM invasion. Increased invasiveness and migration correlates with enhanced expression and activation of MMP-2. Our study provides evidence showing that OPN increases gelatinolytic activity by inducing MT1-MMP expression via activation of the NF-κB pathway. Suppression of MMP-2 by ASMMP-2 reduces the OPN-induced cell migration and ECM invasion. Curcumin blocks OPN-induced MT1-MMP expression and pro-MMP-2 activation. Curcumin, a known anti-inflammatory and anticarcinogenic compound, suppresses OPN-induced cell migration, invasion and induces apoptotic morphology in OPN-treated cells. The mechanism by which curcumin suppresses the OPN-induced effects has also been delineated. Curcumin inhibits MT1-MMP gene expression by blocking signals leading to IKK activation. This in turn inhibits IκBα phosphorylation and NF-κB activation.
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Introduction

Matrix metalloproteinases (MMPs) belong to a family of zinc-dependent endopeptidases that degrade the extracellular matrix (ECM). MMP-mediated matrix degradation is important in several biological processes such as embryogenesis, wound healing, connective tissue remodeling, inflammatory responses, and in pathologies such as arthritis and cancer [1]. Due to matrix-degrading properties, MMPs are highly regulated. Regulation of MMPs occurs on three levels: activation of gene expression, activation of latent zymogens, and inhibition by tissue inhibitors of matrix metalloproteases (TIMPs). The cooperative effects of these three factors exert a tightly controlled regulation of MMPs in normal physiological states. Alteration in all three levels of control has been associated with tumor progression.

MMP activity is regulated by a variety of cytokines, growth factors, hormones, and cellular transformations [2]. In addition to cytokines and growth factors, MMP activity is also regulated by cell microenvironment. Cell-cell, cell-matrix, and extra cellular matrix components have been reported to influence MMPs expression. Vitronectin induces the expression of MMP-2 in melanoma cells [3]. Collagen and elastin induce expression of collagenases and gelatinases by tumor cells or surrounding fibroblasts [4,5]. $\alpha_5\beta_1$ integrin and fibronectin interaction induces MMP-9, MT1-MMP expression in cells cultured in collagen [5,6]. Maquoi *et al*. [7] demonstrated that type IV collagen induces MMP-2 activation in human fibrosarcoma cells . The proteolytic activity of MMP is regulated by being secreted as inactive precursor. Pro-MMPs are activated by cleavage by other proteases found in the extracellular milieu. The most widely accepted model for pro-MMP activation is the cysteine switch model, which involves the destruction of Cys-Zn interaction between the catalytic and prodomain [8]. MMP activity is also regulated by endogenous inhibitors named tissue inhibitors of MMPs (TIMPs) [9].

Since these enzymes can act on several ECM proteins they are involved in basement membrane remodeling, which ultimately

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regulates cell migration and proliferation during cancer cell invasion. MMPs play a critical role in all processes associated with tumor development including invasion, metastasis, and angiogenesis. The signaling pathways that regulate these processes are however, not well defined.

MMP-2 or gelatinase A is secreted as inactive proenzyme, pro-MMP-2. The activation of pro MMP-2 is thought to take place on the cell surface. Pro-MMP-2 is activated by MT1-MMP. This process requires both active MT1-MMP and TIMP-2. The amino terminal of TIMP-2 binds to the catalytic domain of MT1-MMP forming a receptor, and the carboxyl terminal of TIMP-2 then interacts with the haemopexin domain of MMP-2 forming a ternary complex. Cell surface-localized pro-MMP-2 is cleaved by another molecule of active MT1-MMP [10,11]. Coordinated regulation of these proteins is therefore required to regulate the proteolytic activity of MMP-2. Several reports have indicated that increased levels of MMP-2 correlate with the invasive properties of certain tumor cell types [12,13].

Osteopontin (OPN) is a secreted non-collagenous, sialic acid-rich, glycosylated adhesive phosphoprotein [14]. It has a GRGDS cell adhesion sequence at the centre of the single chain, which is highly conserved [15]. It has been shown in various studies to be involved in cell attachment, spreading, intracellular signaling, and cell migration by interacting with integrin receptors [16,17]. A causal role for OPN during tumor progression has been suggested by several studies which demonstrate that it is over-expressed in various carcinomas by either tumor or the surrounding stromal cells like cancer of the colon, duodenum, stomach, breast, lung, prostate, melanoma, bladder, ovary, thyroid, and pancreas [18]. Several highly metastatic-transformed cells synthesize higher levels of OPN than nontumorigenic cells; OPN expression that correlates with metastatic potential has been shown in various studies [19,20]. Furthermore, the role of OPN in tumorigenesis has also been proved by studies using an antisense OPN cDNA construct designed to eliminate secretion of OPN in transformed cells. Cells transfected with antisense OPN construct exhibit reduced ability to form tumor and to metastasize *in vivo* [21,22]. The levels of OPN in blood plasma of patients with metastatic cancer are higher compared with the low levels in normal individuals [23]. All the above data suggest that OPN plays a role in ability of tumor cells to survive and metastasize to secondary sites. However, the biochemical and the molecular mechanisms by which OPN can regulate tumor growth and metastasis to secondary sites is not well understood.

NF-κB is a family of transcription factors, which have been shown to be involved in the control of large number of normal cellular processes such as inflammatory and immune responses, developmental processes, cell growth and apoptosis. In addition, NFκB is activated in several pathological conditions like arthritis, inflammation, asthma, neurodegenerative diseases, heart diseases and cancers. Inappropriate NFκB activity has been reported in several cancers [24–26]. This family of proteins is particularly interesting due to its implication for therapies of diseases like cancer and AIDS. In most cells, NF-κB is present as a latent inactive, IκB-bound complex in the cytoplasm [27]. When a cell receives an extracellular signal, NF-κB rapidly translocates to the nucleus and activates gene expression [28,29]. The exact molecular mechanism by which various extracellular stimuli lead to NF-κB activation is not clear. However, most signals induce the activity of a large multisubunit protein kinase called IκB kinase (IKK) [30]. Active IKK phosphorylates two serine residues in IκBα which targets IκBα for ubiquination and degradation by proteases [27,31]. NF-κB inducible genes play an important role in various disorders, especially cancer. NF-κB induces antiapoptotic genes and protects cancer cells from apoptosis [32,33]. They also induce expression and activation of MMPs, which play a role in ECM degradation and facilitate cell motility and tumor growth [34,35]. Therefore, compounds that block NF-κB activity can be used as a means for inhibiting tumor growth or sensitizing cells to more conventional therapies such as chemotherapy.

Curcumin (Diferuloylmethane) is a major component of turmeric (*Curcuma longa*). In certain countries curcumin has traditionally been used to treat various inflammatory disorders [36,37]. The anti-inflammatory and anticarcinogenic properties of curcumin have subsequently been shown in several reports [38,39]. The mechanisms by which curcumin show these anti-inflammatory and anti-carcinogenic properties are not well understood.

In this study we investigated the mechanism by which OPN facilitates cell motility, ECM invasion, and tumor growth. Since MMP activity is regulated by ECM components, it was speculated that OPN, which is known to be overexpressed in a variety of tumors, might play a role in its regulation. The signaling pathway by which OPN regulates MMP-2 activation has been delineated. There have been reports that curcumin inhibits NF-κB activation induced by several agents [40,41]. We also investigated the effect of curcumin on OPN-induced MMP-2 activation, which showed mediation by NF-κB pathway [34].

Experimental procedures

Zymography for detection of MMP-2 activity

OPN was purified as described previously [34]. B16F10 cells were treated with OPN (0–10 μM) or pretreated with curcumin (0–100 μM) in a serum free medium for 45 min and then incubated with OPN (5 μM) for 12 h at 37°C. Gelatinolytic activity in the conditioned media of B16F10 cells was assessed by zymography. The samples containing equal amounts of total protein were mixed with a sample buffer in the absence of reducing agent and loaded to the zymography-SDS-gel copolymerized with gelatin (0.5 mg/ml). The gels were washed and incubated in incubation buffer (50 mM Tris-HCl, (pH 7.5) containing 100 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% (v/v) Triton-X100, and 0.02% (w/v) NaN_3) for 16 h. The gels were stained with Coomassie blue and destained. Negative staining showed the zones of gelatinolytic activity.

Nuclear and cytoplasmic fractionation and western blot

The nuclear extracts from B16F10 cells were prepared using a modification of the Dignam method [42]. Briefly, the cells were either treated with OPN (5 μM) for 3 h or with curcumin (50 μM) for 45 min followed by OPN (5 μM) for 3 h at 37°C. Cells were scraped, washed in PBS, and resuspended in hypotonic buffer (10 mM Hepes, pH 7.9 containing 10 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT) 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 at 4°C for 5 min. The supernatant was used as cytoplasmic extract. The nuclei were resuspended in nuclear extraction buffer (20 mM Hepes, pH 7.9 containing 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) for 30 min on ice, and centrifuged at 12, 000 $\times$ g for 30 min. The supernatant was used as nuclear extract. The nuclear and cytoplasmic extracts (30 μg) were resolved by SDS-PAGE, and then electrotransferred to the nitrocellulose membrane. The level of NF-κB p65 in both nuclear and cytoplasmic extracts was detected by Western blot analysis using rabbit polyclonal anti-NF-κB p65 antibody.

Immunofluorescence assay

Cells were detached from a plastic tissue culture dish with trypsin-EDTA solution. The cells were resuspended in culture medium and transferred to dishes with sterile cover glasses and grown up to semi-confluency. The cells were then either treated with OPN (5 μM) for 3 h or treated with curcumin (50 μM) and then stimulated with OPN (5 μM) in basal media. The cover glasses were rinsed in PBS, pH 7.4. Cells were fixed with pre-cooled 100% methanol for 10 min, blocked with 5% BSA in PBS pH 7.4, for 30 min, and washed with PBS. The cells were incubated with rabbit polyclonal anti-p65 antibody (diluted 1:100 in 1% BSA in PBS) for 60 min, followed by FITC-conjugated goat anti-rabbit IgG (diluted 1:100 in 1%BSA) for 60 min. The cover slips were mounted using fluorescence mounting media. p65 staining was visualized under a fluorescence microscope.

Western blot analysis

To check whether OPN induced MT1-MMP expression through NF-κB/IκB-α pathways, a super-repressor form of IκBα fused downstream to a flag epitope in an expression vector (pCMV4) was transiently transfected into cells using LipofectAMINE Plus reagent according to the instructions of the manufacturer (Invitrogen). After 48 h, the cells were treated in the absence or presence of OPN (5 μM) and incubated further at 37°C for 16 h. Cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 1% NP-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM iodoacetamide, and 2 mM PMSF) at 4°C for 2 h. The supernatant was collected by centrifugation. In separate experiments, the cells were either treated with 5 μM OPN for 12 h or curcumin (0–100 μM) for 45 min and then with 5 μM OPN for 12 h and the cell lysates were analyzed by SDS-PAGE followed by Western blot analysis using mouse monoclonal anti-MT1-MMP (1:1000 dilution) antibody. As control, the expression of actin was also detected by reprobing the blot with anti-actin antibody.

For IκBα phosphorylation studies, the cells were either treated with 5 μM OPN for 0–3 h or with 50 μM curcumin for 45 min and then with 5 μM OPN for 0–2 h. The cells were lysed in RIPA buffer, and the protein concentrations in cleared supernatants were measured using Bio-Rad protein assay. The supernatants (lysates) containing equal amount of total proteins (50 μg) were resolved by SDS-PAGE and electrotransferred from gel to nitrocellulose membranes. The membranes were incubated with rabbit anti-phospho-IκBα antibody (1:500 dilution) and further incubated with horseradish peroxidase-conjugated anti-rabbit IgG and detected by an ECL-detection system (Amersham Biosciences) according to the manufacturer's instructions. The same blots were reprobed with rabbit anti-nonphospho IκBα (1:500 dilution) or anti-actin (1:1000 dilution) antibody and detected by ECL-detection system.

Cell migration assay

The migration assay was performed using transwell cell culture chambers as described previously [43]. Briefly, a subconfluent monolayer of cells was harvested with trypsin-EDTA and centrifuged at 800 $\times$ g for 10 min. The cells were washed in DMEM containing 0.1% BSA and resuspended in the same media. The cell suspension (5×10^5 cells/well) was added to the upper chamber of a prehydrated polycarbonate membrane filter. Cells in the upper chamber were treated with various concentrations of purified intact OPN (0–10 μM) and the lower chamber was filled with fibroblast-conditioned media which served as a chemoattractant. In order to check whether OPN-stimulated migration was mediated by RGD interaction with integrins, cells were individually treated with anti-αvβ3 antibody (40 μg/ml), or various synthetic RGD-containing peptides for 20 min prior to OPN stimulation. To check the effect of curcumin, an anti-inflammatory and anti-carcinogenic compound on OPN-induced cell migration, a cell suspension was treated with curcumin (0–100 μM) for 30 min at 37°C and added to the upper chamber of a prehydrated polycarbonate membrane. Purified OPN (5 μM) was added to the upper chamber. After incubating at 37°C for 16 h, non-migrated cells were scraped from the upper side of the filter; the filter was fixed in 70% methanol, stained with Giemsa, washed with PBS, pH 7.6. The cells were counted under an inverted microscope. To delineate the role of MMP-2 in OPN-induced cell migration, OPN (10 μM) treated cells were transfected with antisense or sense MMP-2 specific-S-oligonucleotide (AS-MMP-2 or S-MMP-2) and added to the upper chamber, the migration assay was performed under the same conditions as described above. Experiments were performed in triplicate.

ECM invasion assay

ECM invasion assay was done as described previously [44]. The assay was performed in commercially available 24-well plates having two chambers divided by a porous filter. The upper side of the filter is precoated with a layer of artificial basement membrane, Matrigel^{TM} (Collaborative Biomedical). The cells were washed and resuspended in DMEM containing 0.1% BSA. Cells (5×10^5 cells) were either treated with different doses of OPN (0–10 μM), or pretreated with $\alpha v\beta 3$ antibody (40 μg/ml) or synthetic peptides GRGDSP, GPenGRGDSPCA and GRGESP (5 μM) for 20 min followed by treatment with OPN and added to the upper chamber. The lower chamber was filled with fibroblast-conditioned media and incubated at 37°C for 16 h. The non-invading cells and Matrigel from the upper side of the filter were scraped and removed using a moist cotton swab. Cells, which invade to the lower side, were stained with Giemsa. The number of invaded cells was counted under an inverted microscope. For checking the effect of curcumin on OPN-induced invasion, the cell suspension was treated with curcumin (0–100 μM) for 30 min at 37°C and added to the upper chamber of prehydrated polycarbonate membrane. Purified OPN (5 μM) was added to upper chamber. To asses the role of MMP-2 in OPN-induced invasion, OPN (10 μM) treated cells were transfected with antisense or sense MMP-2-specific S-oligonucleotide (AS-MMP-2 or S-MMP-2) and added to the upper chamber; invasion assay was then performed under the same conditions as described above.

Electrophoretic mobility shift assay (EMSA)

The nuclear extracts were prepared either from cells treated with 5 μM OPN alone for 3 h or with curcumin (0–100 μM) for 45 min and then with 5 μM OPN for 3 h. The nuclear extracts (10 μg) were incubated with 16 fmol of ^{32}P-labeled double stranded NF-κB oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) in binding buffer (25 mM Hepes, pH 7.9 containing 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl) with 2 μg of polydeoxyinosinic deoxycytidylic acid (poly dI-dC). The DNA-protein complex was resolved on a native polyacrylamide gel, and analyzed by autoradiography.

NF-κB luciferase reporter gene assay

The semi confluent cells grown in 24-well plates were transiently transfected with a luciferase reporter construct (pNF-κB-Luc) containing five tandem repeats of the NF-κB-binding site using LipofectAMINE Plus reagent. Transfection efficiency was normalized by co-transfecting 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, the cells were treated with varying doses of OPN (0–10 μM) for 6 h or with curcumin (0–100 μM) for 45 min and then with 5 μM OPN for 6 h. The cells were also treated with phorbol myristate acetate (PMA) (50 ng/ml) at 37°C for 6 h as positive control. Cells were harvested in 100 μl passive lysis buffer (Promega). The luciferase activities in 20 μl of the lysates 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 the control were calculated.

IKK assay

The IKK activity was measured as described [45]. The semi-confluent cells were either treated with 5 μM OPN alone for 10 min or with curcumin (50 μM) for 45 min and then treated with 5 μM OPN for 10 min at 37°C. The cells were scraped, washed, and lysed in cold kinase assay lysis buffer (20 mM Tris-HCl, pH 8.0 containing 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM 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 $12,000 \times g$ for 10 min at 4°C. The cell lysates (300 μg) were immunoprecipitated with anti-IKKα/β antibody. Half of the immunoprecipitated samples were incubated with recombinant IκBα (4 μg) in kinase buffer (20 mM Hepes, pH 7.7 containing 2 mM $MgCl_2$, 10 μM ATP, 3 μCi of (γ-^{32}p]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT) at 30°C for 1 h. The kinase reaction was stopped by addition of SDS-sample buffer. The sample was resolved by SDS-PAGE, dried and autoradiographed. The remaining half of the immunoprecipitated samples were subjected to SDS-PAGE and analyzed by Western blot analysis using anti-IKKα/β antibody.

Propidium iodide (PI) staining of cells

The cells grown on sterile glass cover slips were pretreated with 50 μM curcumin for 45 min and then treated in the absence or presence of OPN (5 μM) at 37°C for 6 h. The cells were washed with PBS (pH 7.4), fixed in 1:1 acetone: methanol for 10 min. The cells on the cover slips were dried and treated with PI (50 μg/ml) solution containing RNAase A (20 μg/ml) for 20 min. The cells were washed again, mounted on slides, and visualized under fluorescence confocal microscopy (Ziess).

Results

OPN induces MMP-2 expression and activation, and curcumin suppresses OPN-induced MMP-2 expression and activation

The effect of OPN on pro-MMP-2 production and activation was analyzed by treating B16F10 cells with increasing concentrations of purified OPN (0–10 μM). The conditioned medium was collected and gelatinolytic activity of MMP-2 was detected by zymography. To check whether curcumin suppressed the OPN-induced pro-MMP-2 activation in these cells, the cells

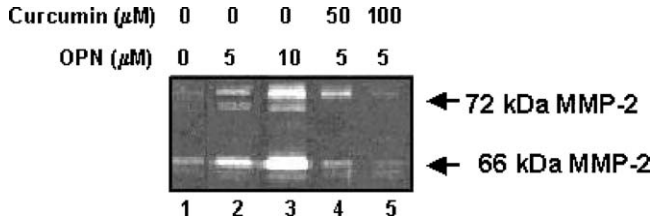

Figure 1. Detection of MMP-2 expression in B16F10 cells by Zymography. The cells were treated with OPN (0–10.0 μM) alone or with varying doses of curcumin (0–100 μM) and then with 5 μM OPN, conditioned media were collected and MMP-2 activity was analyzed by gelatin zymography. lane 1: untreated cells; lane 2: with 5 μM OPN; lane 3: with 10 μM OPN; lane 4: with 50 μM curcumin & 5 μM OPN and Lane 5: with 100 μM curcumin & 5 μM OPN.

were treated with curcumin (0–100 μM) for 45 min and then with 5 μM OPN for 12 h. The conditioned medium was collected and the MMP-2 activity was detected by zymography. The results showed that the OPN induces the expression and activation of MMP-2 in a dose-dependant manner (Figure 1, lanes 2 and 3) compared with a low level in untreated cells (lane1). However, the cells pretreated with 50 μM or 100 μM curcumin followed by treatment with OPN showed drastic reduction of both pro-MMP-2 expression and activation (lanes 4 and 5) indicating that curcumin blocked the OPN-induced pro-MMP-2 activation.

OPN induces MT1-MMP expression by activating NF-κB

The mechanism of activation of pro-MMP-2 involves complex formation of pro-MMP-2 with MT1-MMP and TIMP-2. Therefore, it was checked whether OPN has any role on MT1-MMP expression. The Western blot analysis data indicated that the level of MT1-MMP expression (58 kDa) was 1.5 times higher in OPN-treated (lane 2) cells than in the untreated (lane 1) cells (Figure 2, panel a). It has been previously shown that OPN stimulates NF-κB signaling by interacting with $\alpha v\beta 3$ integrin in endothelial cells [46] and MT1-MMP has an NF-κB binding site in its promoter [35]. It was therefore speculated that NF-κB might be involved in OPN induced MMP-2 activation through induction of MT1-MMP in B16F10 cells. The Western blot of the nuclear and cytoplasmic fractions of OPN-treated or untreated cells revealed that p65 is localized mostly in cytoplasm (Figure 3A, lane 1) as compared with the nucleus (lane 2) in the OPN-untreated cells. However, it translocated from cytoplasm (lane 3) to nucleus (lane 4) in OPN-treated cells. These data were quantified by densitometry and analyzed statistically (Figure 3, lower panel A).

To check whether OPN-induced MT1-MMP expression, through the NF-κB/ IκBα pathway, the cells were transiently transfected with a super-repressor form of IκBα and then treated with OPN. MT1-MMP expression was checked by Western blot analysis. The levels of MT1-MMP in OPN untreated (lane 3) and treated (lane 4) cells were almost identical in the IκBα

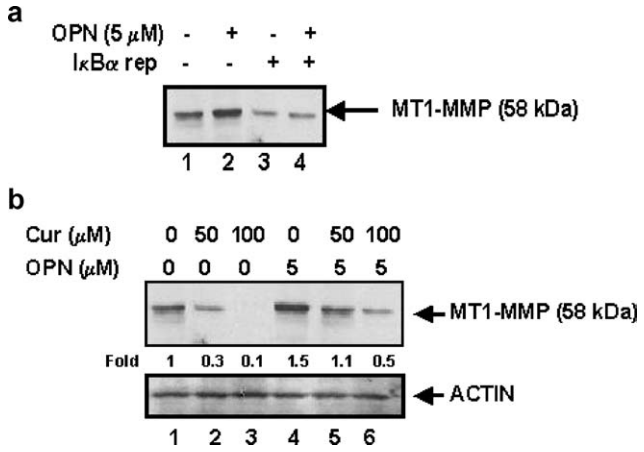

Figure 2. Inhibition of OPN induced MT1-MMP expression by NF-κB super repressor IκBα and curcumin. Panel a: Cells were transfected with super repressor form of IκB-α and then treated in absence or presence of OPN. The cell lysates were used for detection of MT1-MMP by Western blot analysis. Lane 1: untreated cells; lane 2: OPN-treated cells; lane 3: untreated, transfected cells and lane 4: OPN-treated, transfected cells. Note that there is an increase of MT1-MMP expression in OPN-treated cells (lane 2) as compared to control (lane 1) whereas in transfected cells, no significant differences in expression of MT1-MMP are observed in both untreated (lane 3) and OPN-treated (lane 4) cells. Panel b: Cells were pretreated with different doses of curcumin (0–100 μM) and then treated in absence or presence of 5 μM OPN. The cell lysates were used for detection of MT1-MMP by Western blot analysis (upper panel). The same blot was reprobed with anti-actin antibody (lower panel). Lane 1: untreated cells; lane 2: with 50 μM curcumin; lane 3: with 100 μM curcumin; lane 4: with 5 μM OPN; lane 5: with 50 μM curcumin & 5 μM OPN and lane 6: with 100 μM curcumin & 5 μM OPN. The bands were analyzed by densitometry and normalized to actin expression. The fold changes were calculated.

transfected cells (Figure 2, panel a) indicating that IκBα super-repressor could block OPN-induced MT1-MMP expression. These observations suggest that OPN activates the NF-κB signaling pathway leading to induction of MT1-MMP, and increased MT1-MMP expression leads to enhanced activation of pro MMP-2.

Curcumin blocks OPN-induced MT1-MMP expression

The above results indicate that OPN stimulates pro-MMP-2 activation by inducing the expression of MT1-MMP through the NF-κB-mediated pathway. Since curcumin can act as an inhibitor of NF-κB activation, the effect of curcumin on OPN-induced MT1-MMP was checked. The cells were treated with varying concentrations of curcumin (0–100 μM) for 45 min. The cells were also treated with different doses of curcumin (0–100 μM) and then treated with 5 μM OPN for 12 h. Cells were lysed and the lysates containing equal amount of total proteins were separated by SDS-PAGE, and the level of MT1-MMP was detected by Western blot analysis using anti-MT1-MMP

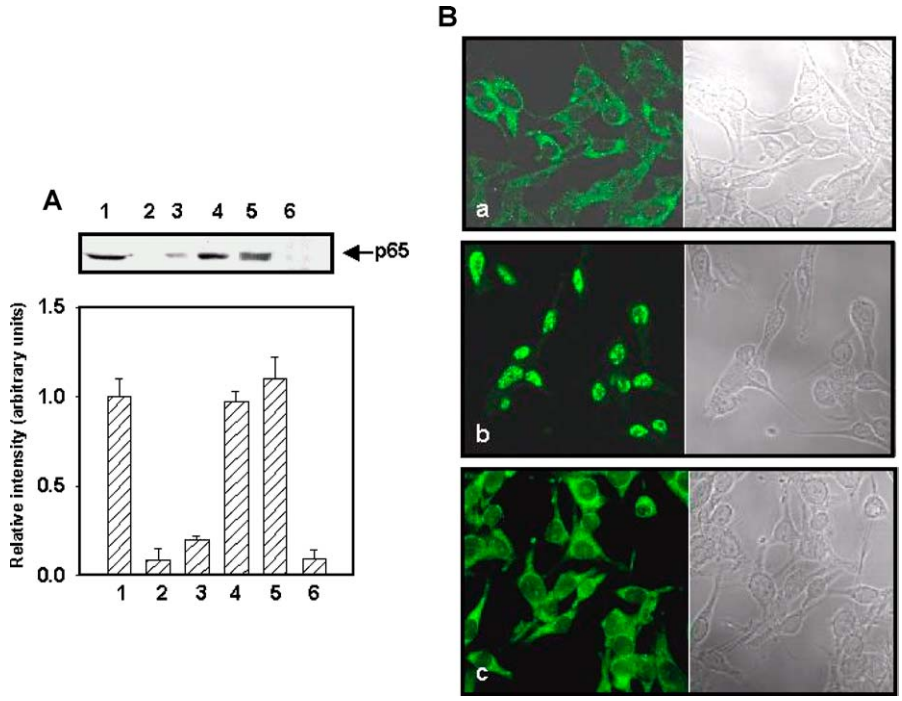

Figure 3. Effect of OPN and curcumin on cellular localization of NF-κB, p65 by immunofluorescence and Western blot analysis. Panel A: Nuclear and cytoplasmic extracts from untreated and treated cells were used for Western blot analysis with rabbit polyclonal anti-NF-κB, p65 antibody. In the untreated cells, p65 was detected in the cytoplasm (lane 1) but not in the nucleus (lane 2). In the OPN treated cells, p65 translocated from cytoplasm (lane 3) into the nucleus (lane 4). The p65 remained in the cytoplasm (lane 5) and did not translocate to the nucleus (lane 6) when the cells were pretreated with curcumin prior to stimulation with OPN. The levels of p65 were quantified by densitometric analysis and is represented in the form of a bar graph (lower panel). Panel B: Cells were treated with 5 μM OPN for 3 h or pretreated with 50 μM curcumin for 45 min followed by treatment with OPN for 3 h. The cells were immune-stained and analyzed under confocal microscopy. In the untreated cells, the majority of p65 was detected in the cytoplasm (panel a) but in the OPN-treated cells, p65 translocated from the cytoplasm to the nucleus (panel b). In contrast, upon treatment of cells with curcumin and then with OPN, the majority of p65 staining was detected in the cytoplasm (panel c).

antibody. There was a significant increase of MT1-MMP expression in OPN-treated cells (Figure 2 upper panel b, lane 4) compared with untreated cells (lane 1) and these data are consistent with the previous results. However, curcumin dose dependently suppressed the OPN-induced MT1-MMP expression (lanes 5 and 6). The MT1-MMP expression was also drastically reduced when increasing concentrations of curcumin alone were used (lanes 2 and 3). The blot was reprobed with anti-actin antibody (lower panel). The bands were quantified densitometrically and normalized with actin. Fold changes with respect to control was calculated. There was at least 1.4- to 3-fold decrease in MT1-MMP expression when the cells were pretreated with 50 μM and 100 μM curcumin respectively prior to the OPN treatment compared with the cells treated with OPN alone.

OPN enhances *in vitro* cell migration and ECM invasion

The effects of OPN treatment on cell migration and ECM-invasion in B16F10 cells was evaluated using established *in vitro* assay systems. Figure 4 shows the dose-dependent increase in cellular migration when increasing concentrations of OPN (0–10.0 μM) were used in the upper chamber. OPN-untreated cells were used as the control (100% migration). Interestingly, pretreatment of cells with GRGDSP or GPen-GRGDSPCA peptides prior to addition of OPN reduced the OPN-induced migration drastically. GRGESP peptide, lacking the RGD cell adhesion sequence, was used as the control and did not show any effect. When cells were pretreated with anti-αvβ3 integrin blocking antibody, prior to the addition of OPN, OPN induced migration was again reduced. Similarly there was a significant increase in ECM-invasion when the cells were treated with increasing concentrations of OPN (0–10 μM). The OPN untreated cells are used as control (100% invasion). However, the OPN-induced ECM-invasion is also suppressed when the cells are individually pretreated with anti-αvβ3 integrin antibody, GRGDSP or GPenGRGDSPCA peptides but not with GRGESP, prior to the addition of OPN (data not shown). These data indicate that OPN-induces migration and ECM-invasion by interacting with its receptor, αvβ3 integrin and the RGD sequence of OPN is involved in both these processes.

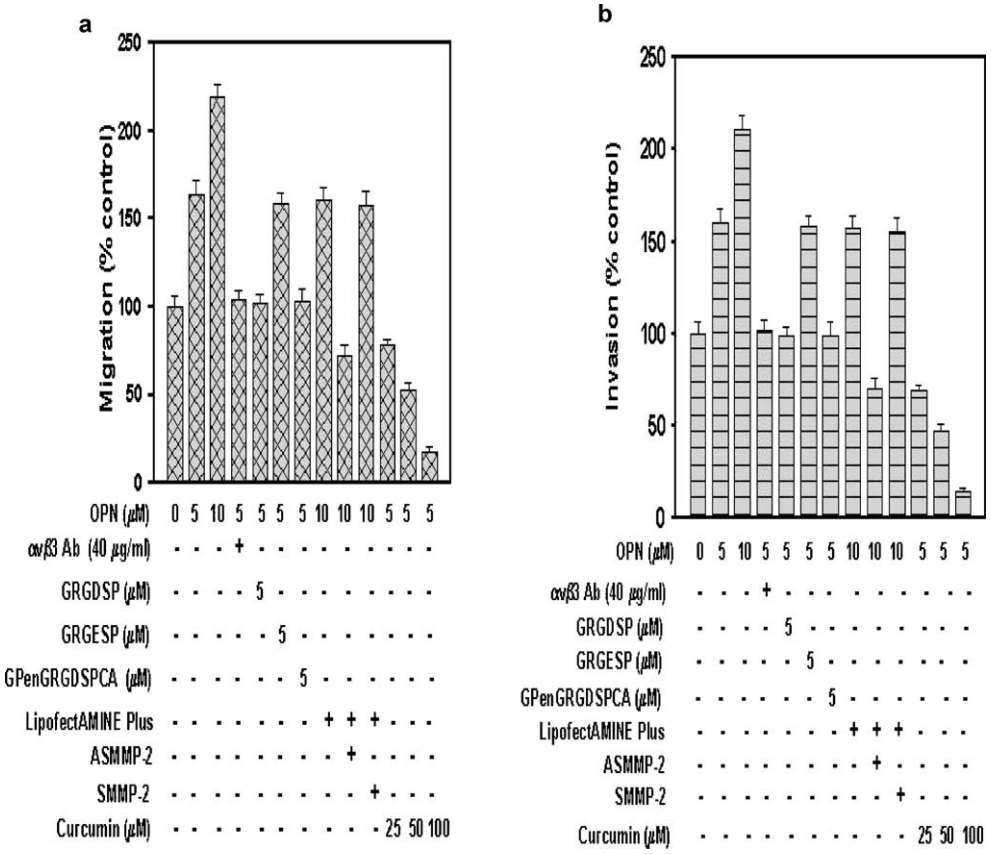

Figure 4. Cell migration and ECM invasion assays. The migration or ECM invasion assays were performed either by using untreated cells (5×10^5 cells/well) or cells treated with different concentrations of purified human OPN (0–10 μM) in the upper chamber. In separate experiments, the cells were pretreated individually with anti-$\alpha v\beta 3$ integrin antibody (40 μg/ml), GRGDSP, GRGESP or GPenGRGDSPCA (5.0 μM of each peptide) or curcumin (0–100 μM) and then treated with OPN (5.0 μM) in the upper chamber. In another experiment, OPN treated cells were transfected with SMMP-2 or ASMMP-2 and used for migration assay. The results are expressed as the mean of three determinations ± SEM.

MMP-2 plays a crucial role in *in vitro* cell migration and ECM-invasion

To assess the role of MMP-2 in cell migration and ECM-invasion, the OPN-treated cells were transiently transfected with MMP-2 specific sense (SMMP-2) or anti-sense S oligonucleotide (ASMMP-2) using LipofectAMINE Plus reagent. The conditioned medium was collected after transfection and the level of MMP-2 was analyzed by zymography. MMP-2 expression was reduced when the cells were transfected with ASMMP-2, but there was no significant difference in MMP-2 expression in cells incubated with LipofectAMINE Plus or transfected with SMMP-2 (data not shown). The transfected cells were used for cell migration (Figure 4, panel a) and invasion (panel b) assays. OPN-induced migration in ASMMP-2 transfected cells was 49% less compared to untransfected cells treated with OPN, but no significant differences were observed in LipofectAMINE Plus or SMMP-2 transfected cells (Figure 4, panel a). Similarly, a reduction in OPN-induced invasion (44%) was observed in ASMMP-2 transfected cells, but no significant changes were noted in LipofectAMINE Plus or SMMP-2 transfected cells (Figure 4, panel b). These results demonstrate that MMP-2 plays an important role in OPN-induced cellular migration and invasion.

Curcumin suppresses OPN-induced *in vitro* cell migration and ECM invasion

To assess the functional effect of inhibition of OPN induced MMP-2 activation by curcumin, cell migration and ECM invasion assays were performed. The cells were pretreated with varying concentrations of curcumin (0–100 μM) and added to the upper chamber. The purified OPN (5 μM) was used in the upper chamber. The results indicated that curcumin suppressed the OPN-induced cell migration in a dose dependent manner (Figure 4, panel a). Similarly, there was a dramatic reduction of OPN-induced ECM-invasion when the cells were pretreated with increasing concentrations of curcumin followed by treatment with OPN (Figure 4, panel b). The results are expressed as the mean of three determinations ± SEM.

OPN induces NF-κB activation and curcumin blocks OPN-induced NF-κB activation

To examine the effect of OPN and curcumin on the cellular localization of p65, the cells were pretreated with curcumin for 45 min and then treated with OPN for 3 h. The cells were fixed, incubated with rabbit anti-p65 antibody, incubated further with FITC-conjugated anti-rabbit IgG, and analyzed under confocal microscopy. The results indicated that in absence of OPN, the majority of p65 was in the cytoplasm (panel a), and in the presence of OPN, the p65 translocated into the nucleus (panel b). Curcumin inhibited OPN-induced nuclear translocation, because most of the p65 was localized in the cytoplasm (Figure 3B).

To examine the effect of OPN and curcumin on NF-κB-DNA binding activity, cells were either treated with 5 μM OPN alone for 3 h or pretreated with different concentrations of curcumin (0–100 μM) for 45 min followed by treatment with OPN (5 μM). Nuclear extracts were prepared and used for EMSA using ^{32}P-labeled NF-κB oligonucleotides. Results in Figure 5 show that OPN induced NF-κB-DNA binding (lane 2) compared with untreated cells (lane1). Curcumin (50 and 100 μM) suppressed the OPN-induced NF-κB-DNA-binding in a dose dependant manner (lanes 3 and 4). The NF-κB specific bands were quantified densitometrically and the fold changes were calculated. The data indicated that there is 4.5-fold increase of DNA-binding in OPN treated cells compared with untreated cells. To show that the band obtained by EMSA in OPN treated cells is indeed NF-κB, the nuclear extracts were incubated with anti-p65 or anti-p50 antibody or both the antibodies in combination and then analyzed by EMSA. There was a shift of NF-κB-specific band to higher molecular weight, when the nuclear extracts were treated with anti-p65, anti-p50 or with both antibodies, suggesting that the OPN-activated complex consisted of p65 and p50 subunits (data not shown). Specificity of binding was also confirmed by incubating the nuclear extract with 100-fold excess of unlabeled oligonucleotide, and the data showed that there is a complete displacement of NF-κB specific band (data not shown).

The effect of OPN on NF-κB transcriptional activity was monitored by luciferase reporter- gene assay. The cells were transiently transfected with NF-κB luciferase reporter construct (pNF-κB-Luc) using LipofectAMINE Plus. The transfection efficiency was normalized by co-transfecting 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 stimulated with increasing concentrations of OPN (0–10 μM). Cells were also pretreated with different doses of curcumin (0–100 μM) and then treated with OPN (5 μM). The cell lysates were used to measure luciferase activity. The data demonstrated that OPN stimulates the NF-κB transcriptional activity and curcumin inhibits the OPN-induced NF-κB activity in a dose-dependent manner (Figure 5, panel b). PMA, as the positive control induces the NFκB activity in these cells. The values were normalized to renilla luciferase activity. The fold changes were calculated and mean $\pm$ S.E. of triplicate determinations are plotted.

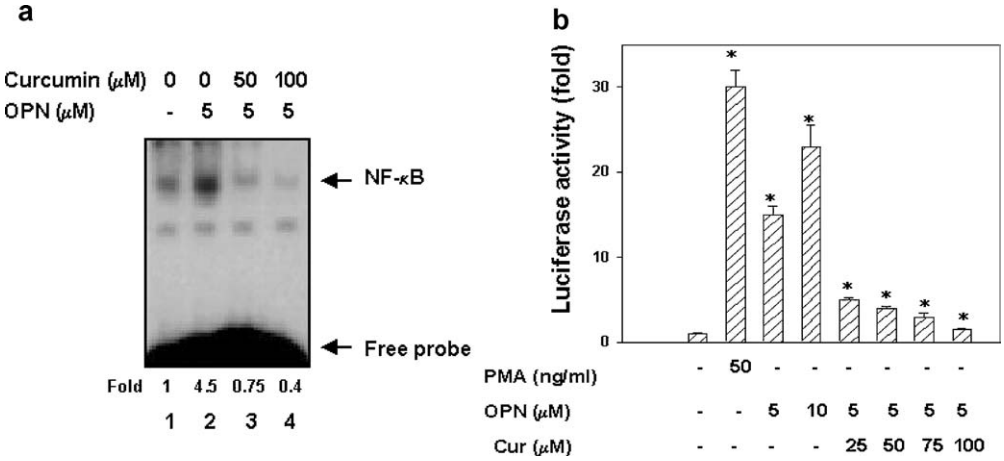

Figure 5. OPN-induces NF-κB-DNA binding and NF-κB transcriptional activity and these effects are suppressed by curcumin. Panel a: The cells were stimulated with 5 μM OPN for 3 h or pretreated with curcumin (0–100 μM) for 45 min and then stimulated with 5 μM OPN for 3 h. Nuclear extracts were prepared and analyzed by EMSA. OPN treatment (lane 2) induces NF-κB binding compared with the untreated cells (lane 1). Curcumin inhibits OPN-induced NF-κB-DNA binding in a dose dependent manner with 50 μM (lane 3) and 100 μM (lane 4) concentrations. The bands were analyzed densitometrically and fold changes are indicated. Panel b: Cells transfected with luciferase reporter construct (pNF-κB-Luc) were either stimulated with PMA (50 ng/ml) or different doses of OPN (0–10 μM) for 6 h or with various doses of curcumin (0–100 μM) for 45 min and then treated with OPN (5 μM) for 6 h. The cell lysates were used to measure the luciferase activity. The values were normalized to renilla luciferase activity. The fold changes were calculated and mean $\pm$ S.E. of triplicate determinations are plotted. The values were also analyzed by student's *t* test (*, $p < 0.001$).

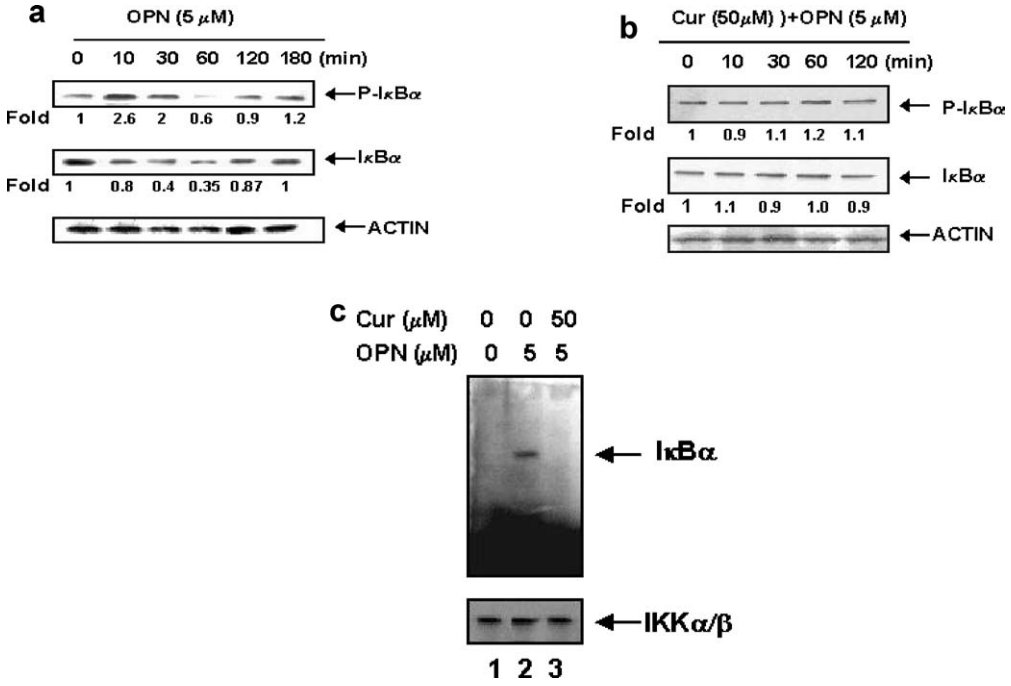

Figure 6. OPN stimulates IκBα phosphorylation and IKK activity and curcumin blocks OPN-induced IκBα phosphorylation and IKK activity. Cells were either treated with 5 μM OPN alone for 0–180 min (panel a) or with 50 μM curcumin for 45 min and then with 5 μM OPN for 0–120 min. The cells were lysed and the lysates were used for Western blot analysis using anti phospho-IκBα antibody (upper panels of a and b). The blots were reprobed with anti-IκBα (middle panels of a and b) or anti-actin (lower panels of a and b) antibody. The blots were analyzed densitometrically and the values were normalized to actin. The relative values of phospho-IκBα or IκBα in terms of fold changes are indicated. Note that the maximum phosphorylation of IκBα occurs at 10 min in OPN treated cells in panel "a" whereas in panel "b", curcumin blocks the OPN-induced phosphorylation and degradation of IκBα. Panel c: Cells were stimulated with 5 μM OPN for 10 min or with 50 μM curcumin for 45 min followed by 5 μM OPN for 10 min. The cell lysates were immunoprecipitated with anti-IKK α/β antibody and used for kinase assay using recombinant IκBα as substrate (upper panel). The immunoprecipitated samples were also analyzed by Western blot analysis using anti-IKK α/β antibody (lower panel). Lane 1: untreated cells; lane 2: with 5 μM OPN and lane 3: with 50 μM curcumin and 5 μM OPN.

OPN stimulates IκBα phosphorylation by inducing IKK activity and curcumin inhibits OPN-induced IκBα phosphorylation and IKK activity

As shown earlier, the OPN-induced NF-κB activity is suppressed by the super-repressor form of IκBα; therefore we examined to see if OPN-induced NF-κB activation occurred through phosphorylation/degradation of IκBα. Cells were treated with OPN (5 μM) for 0 to 180 min and lysed. The lysates containing equal amount of total proteins were resolved by SDS-PAGE and phosphorylated IκBα was detected by Western blot analysis using anti-phospho IκBα antibody. The data demonstrated that OPN induces IκBα phosphorylation in 10 min and the levels of phospho IκBα disappeared in 60 min and reappeared in 120 min (Figure 6, upper panel a). The blot was reprobed with anti-IκBα antibody, and the data indicated that the maximum OPN-induced degradation was observed in 60 min (middle panel a). After that IκBα synthesis was reactivated, possibly by NF-κB, in 180 min (middle panel a). The lack of phosphorylated IκBα at 60 min indicates that the rate of degradation exceeded the rate of IκBα phosphorylation at this time point (upper panel a). As loading controls, the blot was reprobed with anti-actin antibody (lower panel a). In a separate experiment, cells were pretreated with curcumin (50 μM) for 45 min and then stimulated with OPN (5 μM) for 0 to 120 min. The cells were lysed and equal amounts of total protein from the lysates were resolved by SDS-PAGE and analyzed by Western blot analysis using anti-phospho specific IκBα or anti-IκBα antibody as described above. The results indicated that curcumin inhibited OPN-induced IκBα phosphorylation and degradation in these cells (Figure 6, upper and middle panels b). As loading controls, the blot was reprobed with anti-actin antibody (lower panel b). The bands in panels 'a' and 'b' were quantified by densitometry and the values were normalized with respect to actin expression. The fold changes as compared with control were calculated.

Previous reports have indicated that IKK plays a major role in the cytokine-induced phosphorylation of IκBα at serine residues 32 and 36. Therefore, to determine whether OPN controls the IκBα phosphorylation through modulating the activation of IKK and whether curcumin has any effect on IKK

activity, cells were either treated with 5 μM OPN for 10 min or pretreated with 50 μM curcumin for 45 min and then with 5 μM OPN for 10 min. Cells were lysed and immunoprecipitated with anti-IKK α/β antibody. Half of the immunoprecipitated samples were used for kinase assay using recombinant IκBα as substrate. The radiolabeled, phosphorylated IκBα-specific band is detected in OPN-treated cells demonstrating that OPN induces the IKK activity (Figure 6, upper panel c, lane 2). In contrast, IKK activity was undetectable in the untreated or curcumin treated cells (upper panel c, lanes 1 and 3) suggesting that OPN induced IKK activity and that curcumin could block this activity. The remaining half of the immunoprecipitated samples was analyzed by Western blot using anti-IKK α/β antibody. IKK α/β expression was identical in all the samples (lower panel). These data further suggest that curcumin suppressed the OPN-induced NF-κB activation at a step prior to the IκBα phosphorylation.

Curcumin induces apoptotic morphology in OPN treated cells

In order to check whether OPN or curcumin has any role in regulation of apoptosis, these cells were either treated with 5 μM OPN for 6 h or with 50 μM curcumin for 45 min and then with 5 μM OPN for 6 h. The cells were fixed, nuclei were stained with propidium iodide, and photographs were taken under confocal microscopy. OPN alone did not induce apoptotic morphology in these cells (Figure 7, panel b). However, curcumin in the presence of OPN synergistically induced apoptotic morphology within 6 h (panel c). Untreated cells did not show any apoptotic morphology (panel a). These data suggest that curcumin could sensitize OPN-treated cells to apoptosis by selectively inhibiting the signaling pathway for NF-κB activation.

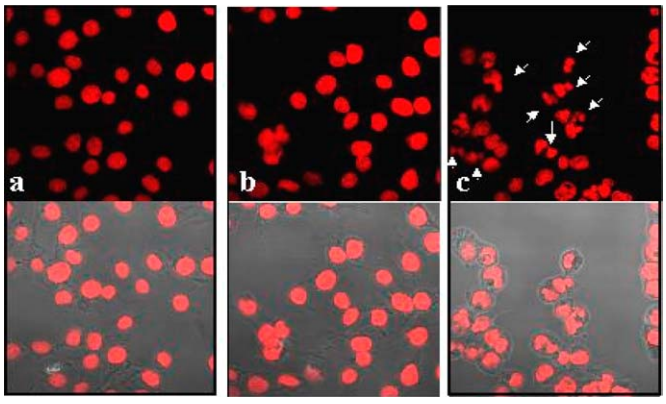

Figure 7. Curcumin induces apoptotic morphology in OPN treated B16F10 cells. Cells were treated with 5 μM OPN for 6 h or with 50 μM curcumin for 45 min and then with 5 μM OPN for 6 h. The cells were fixed and stained with PI. Panel a: untreated cells; panel b: with 5 μM OPN and panel c: with 50 μM curcumin and 5 μM OPN.

Discussion

Rearrangement of ECM by MMPs plays an essential role in cell movement, through alteration of cell-ECM interactions. MMP-2 is an important member of the family by virtue of its ability to act upon a variety of substrates. MMPs are known to be highly regulated by several mechanisms including by the members of the extracellular matrix components. Therefore, it is natural to speculate that ECM components whose expression is changed in transformed cell types may have a role in regulation of MMPs. Osteopontin and its receptors figure prominently in a wide spectrum of malignancies. Therefore, we investigated the potential role of osteopontin in modulation of MMP-2 activity in the murine melanoma cell line, B16F10. We provide evidence to show that OPN upregulates expression of MMP-2 and MT1-MMP. Induction of MT1-MMP correlated with activation of a large fraction of total MMP-2. Data presented here demonstrate that OPN promotes cell migration and ECM invasion and that these effects are dependent on the doses of OPN. Furthermore, MMP-2 activation correlated with the increased cell migration and ECM invasion. Our results have clearly demonstrated the importance of MMP-2 in OPN induced invasion and migration as transfection with MMP-2-specific anti-sense oligonucleotide could suppress these OPN-induced effects.

Previous studies have indicated the role of components of the ECM family in regulating the expression of MMPs. Maquoi *et al*. demonstrated that type IV collagen induces MMP-2 activation in human fibrosarcoma cells [7]. Vitronectin induces MMP-2 and TIMP-2 secretion and enhances invasiveness in B16F1 and B16F10 cells [3]. These data demonstrate that the cell microenviornment plays an important role in regulating its behavior. This study delineates a potentially important mechanism by which increased OPN levels, as they probably occur in case of cancers, lead to MMP-2 expression and activation and cause a substantial enhancement in cell migration and ECM invasion.

Pretreatment of B16F10 cells with anti-αvβ3 antibody blocked OPN-induced cell migration and ECM invasion, suggesting that OPN-enhanced cellular responses occur through interaction with αvβ3 integrin receptor. To check the expression of functional αvβ3 integrin, these cells were surface-labeled and immunoprecipitated with anti-αv and anti-β3 integrin antibodies. These cells were found to be expressing αvβ3 integrin receptor (data not shown). RGD-containing peptides (GRGDSP and GPenGRGDSPCA) suppressed OPN-enhanced cell migration and ECM invasion indicating that OPN interaction with integrin receptor was RGD mediated. Binding and crosslinking experiments with radiolabeled OPN conclusively prove that OPN interacts with its receptor with high affinity and specificity in these cells (data not shown).

The biochemical mechanism by which OPN induces cell migration and ECM invasion by inducing MMP-2 expression and activation has been delineated for the first time. Activation of MMP-2 requires its cell surface localization and cleavage by cell membrane-bound MT1-MMP. TIMP-2 is also required for

this process; it forms a receptor for pro MMP-2 with MT1-MMP. Western blot data showed increased MT1-MMP expression in response to OPN-treatment. Increased MMP-2 activation reflects up-regulation of MT1-MMP in OPN treated cells, implying that OPN facilitates a shift in balance toward increasing proteolytic activity of MMP-2. OPN induced translocation of NF-κB into the nucleus. This correlated with increased MT1-MMP expression. Moreover, OPN induced MT1-MMP expression was blocked in cells transfected with super-repressor form of IκBα, suggesting that OPN induced pro-MMP-2 activation was mediated by activation of NF-κB.

The number of functions attributed to being regulated by the transcription factor NF-κB is rapidly increasing. It is involved in the control of a large number of normal cellular processes such as inflammatory and immune responses, developmental processes, cell growth, and apoptosis. The involvement of most of NF-κB target genes in several disease conditions makes its inhibitors attractive candidates as therapeutic agents. Curcumin (diferuloylmethane) a constituent of turmeric is one such pharmacologically safe, nontoxic compound with known anti-inflammatory and anti-carcinogenic properties. Curcumin blocks activation of NF-κB by TNF-α, PMA, and hydrogen peroxide [41]. Curcumin also blocks cytokine-mediated activation of NF-κB [47]. Therefore, the effect of curcumin on OPN-induced NF-κB activation and its downstream effect such as MT1-MMP expression and pro-MMP-2 activation were analyzed. It was observed that pretreatment of cells with curcumin resulted in inhibition of OPN induced NF-κB activation with concomitant down-regulation of MT1-MMP expression and pro-MMP-2 activation.

The molecular mechanism by which curcumin blocked OPN-induced NF-κB activation was investigated. NF-κB activity is regulated by an endogenous inhibitor IκBα, interaction of NF-κB with IκBα, blocks the nuclear transport signal and keeps it sequestered in the cytoplasm. Following any kind of stimulation, IκB is phosphorylated at serine residues 32 and 36 which lead to its ubiquitination and degradation. The free NF-κB can then translocate into the nucleus and activate the transcription of target genes. Inducible phosphorylation of NF-κB is mediated by a multisubunit complex of kinase, IKK [49]. Our results show that inhibition of OPN-induced NF-κB activity by curcumin involved blocking of OPN-induced IκB-α phosphorylation through inhibition of IKK activity. It is shown that OPN induced NF-κB-mediated MT1-MMP expression and MMP-2 activation. This correlated with enhanced cellular migration and ECM invasion. Moreover, pretreatment of cells with curcumin could inhibit OPN-enhanced migration. Other investigators have shown in the past that OPN ligation to its αvβ3 integrin receptor protected the endothelial cells from apoptosis through NF-κB pathway [46]. This and other reports which show NF-κB induces anti-apoptotic genes implicates NF-κB as an important regulator of cell growth.

Use of natural products is emerging as an alternative to traditional medicines in treatment of cancer. These alternate approaches are relatively non-toxic. However, limited scientific evidence regarding their effectiveness and a lack of mechanistic understanding of their action has led to these compounds not being used in mainstream medicine. Curcumin is a non-toxic natural product that has been used as a food additive [49]. The non toxicity is proved by its consumption by humans in several countries. Curcumin is also shown to be a non-mutagenic compound [50]. Our results indicate that pretreatment of cells with curcumin drastically reduced the cell viability (data not shown) and also induced apoptotic morphology in OPN treated cells. Enhanced proliferation rates and resistance to apoptosis-inducing signals are important factors contributing to tumor growth; therefore antiproliferative and apoptosis inducing property of curcumin and its ability to inhibit NF-κB signaling could prove useful in control of various cancers.

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