Osteopontin Promotes Integrin Activation through Outside-In and Inside-Out Mechanisms: OPN-CD44<sub>V</sub> Interaction Enhances Survival in Gastrointestinal Cancer Cells

Jia-Lin Lee, Mei-Jung Wang, Putty-Reddy Sudhir, Gen-Der Chen, Chin-Wen Chi, and Jeou-Yuan Chen

Abstract

Osteopontin (OPN) and splice variants of CD44 (CD44<sub>V</sub>) have independently been identified as markers for tumor progression. In this study, we show that both OPN and CD44<sub>V</sub> are frequently overexpressed in human gastric cancer and that OPN-engaged CD44<sub>V</sub> ligation confers cells an increased survival mediated through integrin activation. First, we show that OPN treatment confers cells an increased resistance to UV-induced apoptosis. The OPN-mediated antiapoptosis is dependent on the expression of the variant exon 6 (V6)- or V7-containing CD44 as shown by overexpression of individual CD44<sub>V</sub> in gastric AZ521 cells that express no or very low level of endogenous CD44 and by knockdown of the constitutively expressed V6-containing CD44 isoforms in colon HT29 cells. Although OPN also interacts with RGD integrins, OPN-RGD sequence is dispensable for OPN-mediated antiapoptosis. OPN-induced antiapoptosis is mainly attributed to the engagement of CD44<sub>V</sub> isoforms and the relay of an inside-out signaling via Src activity, leading to robust integrin activation. Furthermore, OPN-elicted antiapoptosis was observed when cells were plated on fibronectin but not on poly-D-lysine, and preincubation of cells with anti-integrin antibody to block integrin-extracellular matrix (ECM) interaction or ectopic expression of the dominant-negative forms of focal adhesion kinase to block ECM-derived signal abolished OPN-induced survival, suggesting that OPN-elicted antiapoptotic function is propagated from matrix transduced by integrin. Taken together, we showed that OPN-CD44<sub>V</sub> interaction promotes ECM-derived survival signal mediated through integrin activation, which may play an important role in the pathogenic development and progression of gastric cancer. [Cancer Res 2007;67(5):2089–97]

Introduction

Homeostasis in normal tissue is regulated by a balance between proliferative activity and cell loss by apoptosis (1, 2). Attachment to extracellular matrix (ECM) is essential for survival and growth of normal adhering cells, whereas cancer cells are able to abrogate this requirement. Several growth factors and cytokines play pivotal roles in the regulation of growth and survival of neoplastic cells through affecting integrin-mediated adhesion to ECM. Osteopontin (OPN), a secreted noncollagenous, sialic acid-rich, phosphoprotein and also a member of the SIBLING family, functions as both an ECM component and a cytokine signaling through the binding to two cell adhesion molecules: integrin and CD44 (3, 4). OPN was initially discovered as an inducible marker of transformation of epithelial cells and later shown to be frequently overexpressed in many human cancers (3). The findings that OPN expression correlates with tumor progression in cancers of breast (5), stomach (6), lung (7), prostate (8), liver (9), and colon (10) and that OPN concentration in the plasma of patients with metastatic disease is significantly higher than that in normal sera (11, 12) implicate its role in the regulation of tumor cell migration and metastasis. In fact, numerous studies in cultured cells have shown that OPN expression renders cells more tumorigenic and/or metastatic (8). In contrast, down-regulation of OPN expression by antisense approach reduced cell growth in soft agar and in mice as primary tumors or experimental metastasized foci (3).

The mechanisms by which OPN may enhance malignancy are still unclear. In vitro studies have shown that OPN has multifunctional properties in promoting cell adhesion, cell migration, and cell survival (3). Although many details pertaining to the exact mechanisms remain to be elucidated, OPN functions through its interaction with the integrin and CD44 families of cell surface receptors. OPN contains at its NH<sub>2</sub>-terminal region the RGD tripeptide sequence that can be bound by RGD-dependent integrins (13). OPN also interacts with α<sub>9β<sub>1</sub></sub> and α<sub>9β<sub>1</sub></sub> integrins through a cryptic site of sequence SVVYGLR adjacent to the RGD motif, which is thought to be revealed on thrombin cleavage (14, 15). The COOH-terminal fragment of OPN binds directly to CD44 variant isoforms. CD44, a member of the immunoglobulin superfamily, is expressed in various isoforms with extended extracellular domains by alternative RNA splicing. CD44 binds hyaluronate in the ECM to maintain tissue/organ structure, promote cell aggregation, and mediate cell movement (16). Up-regulation of one or more CD44<sub>V</sub> has been implicated in the progression of a variety of tumors (17). CD44<sub>V6</sub> has been identified as a metastatic marker in lymphoma, hepatocellular, breast, lung, pancreatic, and colorectal cancers (18, 19). In gastric cancer, V6 is up-regulated and associated with tumor progression (20). Functional studies have shown that OPN can interact specifically with CD44 V6 or V7 (4, 21), and ligation of CD44<sub>V</sub> by OPN promotes chemotaxis and adhesion of fibroblasts, T cells, and bone marrow cells (22, 23).

Several signaling pathways are activated by OPN that may contribute to tumor progression and metastatic behavior. On binding to CD44, OPN confers interleukin-3-mediated or granulocyte macrophage colony-stimulating factor-mediated survival
advantage in murine B cells through activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B pathway (24). OPN-mediated ligation of integrin β3 has been shown to provide an increased survival in rat aortic endothelial cells through activation of nuclear factor-κ-B (NF-κ-B; ref. 25). Several studies have shown that OPN acts in concert with several growth factors, including hepatocyte growth factor (26) and epidermal growth factor (27), to induce malignant properties. β3-containing integrins have been shown to cooperate with CD44 variant isoforms to promote OPN-mediated cell motility and chemotaxis in rat pancreatic carcinoma cells (21). In this study, we have attempted to dissect mechanisms that relay OPN/CD44-mediated cellular effects, particularly on cell survival, for further understanding the OPN/CD44-mediated signaling implicated in tumor formation and progression. Our results showed that treatment with baculovirus-produced OPN provides gastric cells increased ability to withstand apoptotic insults through ECM-derived survival signals, and this survival effect was mainly attributed to the binding of OPN to CD44, and the relay of an inside-out signaling, leading to integrin activation.

Materials and Methods

Antibodies and reagents. The hybridomas Hermes-3 and FW11-24-17-36 (anti-CD44v9) were from the American Type Culture Collection (ATCC, Manassas, VA). Mouse monoclonal antibody (mAb) against human CD44v6 (2F10) was from R&D Systems (Minneapolis, MN). Antibodies for integrin β3 (P4C10 for blocking and B3B11 for flow cytometry), αv (AV1), αvβ3 (LM609), αvβ5 (P1F6), and αvβ5 (JBS5) were from Chemicon (Temecula, CA). The mAb HUTS-21 for activated β3 integrin was from BD Biosciences Pharmingen (San Diego, CA). Alexa Fluor 488–conjugated and Alexa Fluor 594–conjugated anti-mouse and anti-rabbit IgG were from Molecular Probes (Eugene, OR). The synthetic peptide GRGD was purchased from Sigma (St. Louis, MO).

Preparation and purification of OPN. Recombinant OPN was produced using the BaculoGold Expression System (BD Biosciences Pharmingen). In brief, the full-length cDNA of human OPN was amplified by PCR from human placenta cDNA and subcloned into the DS Red-encoding baculovirus transfer vector pABhRpB (a gift from Y.-C. Chao, Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan) to yield the OPN-(His)6-encoding pABhRpOPN plasmid. On cotransfection of pABhRpOPN with BaculoGold viral DNA into Sf9 cells, the recombinant OPN-producing baculovirus was generated. High Five insect cells were infected with the recombinant virus at a multiplicity of infection of 5 in Express Five medium (Invitrogen Corp., Carlsbad, CA), culture supernatant was collected after 3 to 4 days, and OPN was purified by Ni-NTA resin (Qiagen, Valencia, CA).

Constructs, cell culture, and transfection. Human gastric adenocarcinoma AZ521 and AGS, non–small cell lung cancer H1299, colorectal cancer HT29, and human embryonic kidney 293 cells were from ATCC. The CD44v6 cDNA was amplified by PCR from H1299 cDNA and subcloned into pCDNA3.1−/−/Myc-His vector to generate pcDNA-CD44v6. Plasmids pcDNA-CD44v6, pcDNA-CD44v6-KRAS pcDNA-CD44v6, pcDNA-CD44v7, pcDNA-CD44v5−, and pcDNA-CD44v7− were constructed by PCR amplification of the specific CD44 variant exons from AGS cDNA and subcloned the PCR fragments into pCDNA-CD44v6 at appropriate sites. The resultant plasmids were sequence verified. The vectors encoding focal adhesion kinase (FAK)-related nonkinase (FRNK) and FAK (Y397F) were kind gifts from Dr. T.-H. Leu (Department of Pharmacology, National Chung-Kung University, Tainan, Taiwan). Plasmid encoding Src (K297D) has been described previously (28). Transient transfection was done using the LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s protocol. AZ521/Mock and AZ521/CD44 cell clones were established by transfection of AZ521 cells with pcDNA3.1 and the respective pcDNA-CD44 plasmids by electroporation method followed by selection of G418-resistant clones as described (29).


Results

Elevated expression of OPN and CD44 in gastric cancer. By cDNA microarray analysis, we have identified OPN as one of the genes overexpressed in gastric cancer, and we have shown that treatment with baculovirus-produced OPN provides gastric cells increased ability to withstand apoptotic insults through ECM-derived survival signals, and this survival effect was mainly attributed to the binding of OPN to CD44 and the relay of an inside-out signaling, leading to integrin activation.

RNA isolation, reverse transcription-PCR, and real-time PCR. Gastric adenocarcinoma and matching nontumor mucosal tissues were obtained from patients who underwent surgery at the Veterans General Hospital (Taipei, Taiwan). Informed consent was obtained from each patient. Tissues were snap frozen immediately following resection. Total RNA was prepared from homogenized tissues and subjected to reverse transcription-PCR (RT-PCR) as described (29, 30). PCR was done using primers for CD44 (sense primer, 5′-GACGAAAGACTCCCTGGAT-3′; antisense primer, 5′-CTT-CTTGACCTCCCATGTG-3′; Genbank accession no. NM_000610) and OPN (sense primer, 5′-GGATCCCTCCTAATCAGGATCAAGAAGCTCCCTGACATC-3′; antisense primer, 5′-AAGCTTGGCTTGAAGATGTTCCCGGG-3′; Genbank accession no. NM_000582), respectively.

Quantitative real-time PCR was carried out as described (29). The threshold cycles (Ct) were recorded for all samples for both the target gene and the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Melt curve analysis was done for each run. Relative gene expression of the target gene was calculated as ∆∆Ct, determined by subtracting the Ct of reference gene from the Ct of target gene. Differential expression of the target gene in tumor versus nontumor mucosal samples was shown as ∆∆Ct, determined by subtracting the ∆Ct of tumor sample from the ∆Ct of the matching mucosal sample.

Knockdown by short hairpin RNA. The short hairpin RNA (shRNA) vectors were constructed by annealing synthetic DNA oligonucleotide primers (CD44v6 sh_S, 5′-GATCCCCCACTGCTTCACTGTCTTACAAAGATGAGAAGAGAGTCTTACTCTGGTTATTGA-3′; CD44v6 sh_AS, 5′-AGCTTTAAAAAGCCAACTCCTGAGTACATCTCTTGTAATTGAATGACTACAGGAGTTGCGCCGG-3′; the sequences corresponding to nucleotides 6–24 of CD44 V6 exon italicized) followed by ligation into pSuper vector driven by the polymerase III H1-RNA gene promoter. The pSuper-CD44v6 sequence was verified by sequence analysis. After transfection of HT29 cells with pSuper-CD44v6, pSuper control vectors, cells were cultured in medium containing 400 μg/mL G418 for 3 to 4 weeks, and stable clones were selected and examined for the expression of V6-containing CD44 by Western blotting.

Flow cytometric analysis. Subconfluent AZ521/CD44v6 and HT29 cells were treated with OPN or OPN(RGE) for 1 h at 37°C and incubated with isotype IgG or HUTS-21, labeled by Alexa Fluor 488–conjugated secondary antibody, and subjected to flow cytometric analysis using FACScalibur (BD Biosciences). Cells preincubated with 2 mmol/L MnCl2 were included as a positive control. In some experiments, cells were pretreated with RGD peptide (10 μmol/L) and/or anti-CD44v6 antibody (2F10) for 1 h at 37°C before the incubation with OPN.

Adhesion assay. Adhesion of cells to plates coated with 20 μg/mL OPN, 10 μg/mL fibronectin, 2 mg/mL poly-γ-hydroxy, or 1% bovine serum albumin (BSA) in PBS was assessed as described previously (31). In some experiments, cells were preincubated with 5 μg/mL of blocking antibodies [anti-integrin αvβ3 (PAC10), αvβ3 (PAC10), or anti-CD44ab] or 10 μg/mL of RGD peptide for 1 h at 37°C and subjected to adhesion assays. The reference value for 100% attachment was obtained by seeding cells on plates precoated with 20 μg/mL fibronectin. Cells were incubated for 3 h at 37°C under tissue culture condition followed by immediate fixation, and approximately 90% to 100% of input cells were recovered.

Apoptosis assay. After UV irradiation, cells were harvested after the designated time, stained with propidium iodide, and subjected to flow cytometric analysis of sub-G1 apoptotic fractions. To test the role of integrin, cells were replated on fibronectin-coated or poly-γ-hydroxy–coated dishes in medium with or without added OPN for 4 h. Cells were UV irradiated and harvested in 48 h for flow cytometric analysis. Alternatively, cells were pretreated with blocking antibody against integrin β3 (PAC10) for 1 h at 37°C before OPN treatment.

Statistical analysis. Statistical analysis of data was done by Student's t test using SigmaPlot software. Difference was considered to be statistically significant at P < 0.05.
lead markers whose expression is altered in human gastric cancer (Fig. 1A, left). OPN is frequently expressed at elevated levels in a variety of human cancers, including gastric cancer. The gastric cancer microarray data (32) in the Stanford Microarray Database also confirmed increased expression of OPN in gastric cancer compared with the nonneoplastic gastric mucosa ($P < 0.0001$, Mann-Whitney $U$ test; Fig. 1A, right). Figure 1B (top) shows the real-time PCR analysis of OPN expression in primary gastric cancer, showing that 75% (six of eight) gastric cancers displayed increased expression of OPN. In these gastric cancer samples, we also examined the expression of CD44, the major cell surface receptor for OPN (Fig. 1B, bottom). The standard CD44 isoform (CD44H) was readily detected and expressed at similar levels in gastric cancer and in the matching nontumorous tissues, whereas the epithelial-specific CD44 isoform (CD44E or CD44V6-8) and many larger variant isoforms, particularly the CD44V6-10, were expressed at elevated levels in gastric cancer compared with the nontumorous mucosa. It was noted that many of the gastric cancers that express higher level of OPN also express increased levels of CD44 variant isoforms.

**OPN protects cells from UV-induced apoptosis dependent on the expression of CD44 variant isoforms.** To determine whether OPN promotes tumorigenesis and progression of epithelial-derived cancers, we examined the response of colon cancer HT29 cells and gastric cancer AZ521 cells for their response to OPN-mediated cellular effects, particularly on cell survival. HT29 cells express high levels of endogenous CD44s and other variant isoforms, whereas AZ521 cells display very low or undetectable CD44 (Supplementary Fig. S1). Subconfluent cells were treated with or without OPN and subjected to UV irradiation, and apoptosis was followed by flow cytometric analysis. As shown in Fig. 2A, UV irradiation induced a time-dependent increase in the number of HT29 and AZ521 cells undergoing apoptosis. Treatment with OPN significantly suppressed UV-induced apoptosis in HT29 but had no effect on AZ521 cells. To corroborate that OPN-mediated antiapoptotic effect is mediated through CD44, AZ521
CD44S, are sensitive to UV-induced apoptosis, and OPN treatment formed cells (Fig. 2C) tested whether OPN provides survival advantage in nontransgenic cells stably expressing designated CD44 variant isoforms. Subconfluent HT29 and AZ521 (A), AZ521 clones harboring control plasmid or plasmids encoding specific CD44 isoforms (B), and HEK293 (C) cells were cultured in serum-free medium for 24 h and incubated with or without added OPN (10 μg/mL) for 4 h. Cells were then UV irradiated at 90 J/m² for HT29 and AZ521 cells or 130 J/m² for HEK293 cells) and harvested for apoptosis assay by flow cytometric analyses of sub-G1 fractions at designated time.

As shown in Fig. 2B, the control and all CD44-expressing cells displayed a time-dependent apoptosis after UV irradiation, whereas OPN treatment conferred a more resistant phenotype toward UV-induced apoptosis in the AZ521/CD44V6-10 cells but not others. We next tested whether OPN provides survival advantage in nontransformed cells (Fig. 2C). HEK293 cells, which exclusively express CD44s, are sensitive to UV-induced apoptosis, and OPN treatment offers little survival advantage. Importantly, ectopic expression of CD44V6-10 rendered HEK293 more resistant to UV-induced apoptosis in the presence of OPN.

**OPN-elicited cell survival is correlated to its interaction with CD44V.** To dissect how CD44 mediates OPN-elicited cellular effects, we assessed the interaction of OPN and CD44 by determining the binding of AZ521 cells expressing designated CD44 isoforms to OPN-coated plates. It has been shown that OPN binds to CD44V6 and possibly CD44V7 isoforms and enhances the migration of rat fibrosarcoma cells (21). To ascertain whether the V6- or V7-encoded sequences account for the CD44 structural prerequisite for OPN-elicited survival effect, AZ521 cell clones expressing CD44V6, CD44V7, CD44V6-10, CD44V7-10, CD44V6-7, CD44V6, and CD44V7 were established and cell binding assays were done (Fig. 3A). As shown, cells expressing CD44 variant isoforms containing either the V6- or V7-encoded sequences adhered to OPN with markedly increased affinity compared with the control cells, suggesting that the increased adherence is dependent on the expression of CD44V. Furthermore, the increased adherence was reversed in AZ521/CD44V6 cells when cells were pretreated with blocking antibody against V6 (Fig. 3B). The binding of OPN to RGD integrins also showed that it was marginally elevated in the AZ521/Mock, AZ521/CD44V6, and AZ521/CD44V7 cells as compared to their binding to BSA (Fig. 3A) and that preincubation with excess amount of RGD peptides completely abolished this binding (Fig. 3B). These results showed that cell binding to OPN is mediated through both CD44-dependent and CD44-independent (integrin dependent) mechanisms. In line with the increased OPN-binding ability in cells expressing CD44 isoforms containing the V6- or V7-encoded sequences, these cells also displayed a more resistant phenotype to UV-induced apoptosis on OPN treatment (Fig. 3C). The fact that OPN-elicited survival was significantly higher in AZ521 cells that express V6- or V7-containing CD44 isoforms (B) compared with AZ521/Mock, AZ521/CD44V6, and AZ521/CD44V7 cells highlights the important role of CD44-dependent but not the RGD-dependent interactions in this event. In line with this notion, OPN(RGE), in which the RGD sequence was mutated to RGE, conferred HT29 cells increased resistance to UV-induced apoptosis as the wild-type OPN did (Fig. 3D).

To corroborate that the antiapoptotic effect of OPN is mediated through CD44V, we studied the effect of targeted knockdown of CD44V expression on OPN-mediated antiapoptosis. We chose to knockdown the expression of V6-containing CD44V mRNA in HT29 cells because these cells express largely the V6-containing variant isoforms. Knockdown was achieved by transfection of a pSuper plasmid carrying the shRNA sequence targeted to CD44V6 region (pSuper-V6) and pSuper alone as a control. After selection, four HT29 shRNA (carrying the shRNA for V6-containing CD44) and two HT29 shRNA (carrying the pSuper) clones were obtained and assessed for OPN-mediated antiapoptosis. The expression of V6-containing CD44 was fully eliminated in HT29 shRNA clones 12 and 31 and reduced by 60% in clones 22 and 27 in comparison with that in the control HT29 shRNA clones C1 and C2. Targeted knockdown of the expression of V6-containing CD44 isoforms effectively reversed OPN-mediated antiapoptosis in HT29 cells in correlation to the reduced levels of V6-containing proteins (Fig. 3D).

**Synergistic effect of CD44 and integrin on cell binding to OPN.** As described above, cells expressing CD44 that contain V6- or V7-encoded sequences exerted significantly elevated adhesion to OPN through both CD44-dependent and CD44-independent (integrin-dependent) manners. We further assessed the contribution of CD44, integrin, or both in combination with OPN using AZ521/CD44V6 cells as a model. As shown in Fig. 4A, CD44-mediated cell binding to OPN, as measured by incubating cells with OPN in the presence of RGD peptides to block integrin-dependent binding, was enhanced commensurate with the
increased levels of CD44 expressed, whereas integrin-mediated binding, which was measured in the presence of anti-CD44 antibody to block CD44-dependent binding, remained marginal and unchanged. In the absence of blocking reagents, significantly, cell binding to OPN was dramatically increased as a function of the levels of CD44 expressed. Similarly, CD44 and integrin also work cooperatively to promote cell adhesion to OPN when incubating with increasing concentrations of OPN (Fig. 4B). These data suggested that CD44, which was shown above to play a significant role in facilitating cell adhesion to OPN, may engage a synergistic effect to promote integrin-mediated cell adherence to OPN. In agreement, pretreatment of cells with blocking antibody against RGD integrins [e.g., β1 (P4C10) and αv] intercepted this effect and limited the ability of the cell to bind OPN solely to the OPN-CD44 interaction (Fig. 4C).

**OPN-mediated CD44 ligation induces integrin activation.** An increase in ECM-integrin interaction could reflect either mobilization of more integrins to the cell surface or increased binding affinity as well as valency of the integrins already present. Thamilselvan and Basson (33) have reported that ECM-integrin interaction stimulated adhesion mediated through integrin activation without changing integrin surface expression. We further examined the process of integrin activation in OPN-mediated survival. The activation of β3 integrin was assessed by flow cytometric analyses using mAb (HUTS-21) specifically recognizing the active conformer of β3 integrin. As shown, robust activation of β3 integrin was observed in the AZ521 (Fig. 5A) and HT29 (Fig. 5B) cells expressing CD44 isoforms containing V6 or V7 sequences. On the other hand, low level of activated β3 integrin was detected in the AZ521/Mock, AZ521/CD44S, and AZ521/CD44E cells (data not shown). Therefore, our data showed that binding of OPN to CD44V efficiently promoted integrin activation. In the absence of the OPN-RGD sequences, OPN(RGE) exhibited similar effect to induce integrin activation (Fig. 5B). In addition to the CD44-dependent activation, RGD integrins are also activated on engagement by OPN in a CD44-independent manner. Preincubation with anti-V6 antibody greatly suppressed OPN-elicited CD44-dependent activation of integrin β1 in AZ521/CD44V6 cells to the level observed in the AZ521/Mock cells (Fig. 5C). The observed moderate level of CD44-independent integrin activation was further suppressed to basal level by the incubation with RGD peptides (Fig. 5C). On OPN treatment, the increased level of integrins with active conformer

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**Figure 3.** Engagement of CD44 containing exon V6- or V7-encoded sequences by OPN enhances cell survival. **A,** binding of CD44-expressing cells to OPN. Subconfluent AZ521 cells (top panel) and HT29 cells (bottom panel) were incubated in serum-free medium for 24 h and replated on dishes precoated with OPN (20 μg/mL) or 1% BSA and allowed to adhere for 30 min. The percentage of cell adhesion was calculated as described in Materials and Methods. **B,** cell binding to OPN in the presence of RGD peptides and anti-CD44 antibody. Subconfluent AZ521/CD44 transfectants were trypsinized, pretreated with RGD peptide (10 μM) or anti-CD44 antibody (10 μg/mL) for 1 h at 37°C, and replated on dishes precoated with OPN for 30 min, and cell adherence was measured. **C** and **D,** OPN-mediated survival in CD44-expressing cells. Subconfluent AZ521/CD44 clones (C) and HT29 (D) cells were incubated in serum-free medium with or without added OPN or OPN(RGE) for 4 h and subjected to UV irradiation followed by apoptosis assay as described in the legend of Fig. 2. **E,** knockdown of the expression of V6-containing CD44 suppresses OPN-mediated survival. HT29 cells were transfected with CD44 V6-specific shRNA (pSuper-V6) or control shRNA (pSuper), and individual cell clones harboring integrated pSuper-V6 and pSuper control were selected. **Top,** immunoblot analyses of the selected cell clones using anti-V6 antibody. The subconfluent HT29 cell clones were subjected to UV irradiation at 90 J/m² in the presence and absence of OPN and subjected to apoptosis assay.
unchanged in HT29 and AZ521/CD44V6 cells after incubation with OPN for 24 h (data not shown).

A pretreated with blocking antibody (5 μg/mL), or a control IgG in cell binding assay. C, cell binding to OPN is suppressed by anti-integrin antibodies. Subconfluent AZ521/CD44V6 cells were pretreated with blocking antibodies (5 μg/mL) against integrins αv, β1 (P4C10), αvβ3, or αvβ5, for 1 h at 37°C and replated on OPN (20 μg/mL) or 1% BSA for 30 min, and cell adhesion was determined. Columns, mean of three independent experiments; bars, SD. **, P < 0.01, Student's t test.

was not due to the increased levels of integrins expressed on cell surface, as the expression of α5, αv, β1, and β3 integrins remained unchanged in HT29 and AZ521/CD44V6 cells after incubation with OPN for 24 h (data not shown).

In recent studies, Src kinase, protein kinase C (PKC), PI3K, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK), and ERK have been implicated in the regulation of integrin activity (34–36). We further examined the potential signaling pathway that may mediate CD44-induced integrin activation. AZ521/CD44V6 cells were incubated with RGD peptides to block the interaction of OPN and RGD integrins and, in the same time, treated with PP2, a Src family kinase inhibitor, as well as inhibitors specific to PI3K (LY294002 and wortmannin), MAPK (PD98059), PKC (GF109203X), and NF-κB (curcumin) followed by the incubation with OPN, and our results showed that the treatment with PP2 significantly blocked OPN-induced integrin activation (Fig. 5D), whereas inhibitors to PI3K, MAPK, PKC, and NF-κB had little effect (data not shown). Furthermore, ectopic expression of a dominant-negative Src (K297D) also blocked OPN-induced integrin activation (Fig. 5D).

These data suggested that OPN-CD44α interaction relays an inside-out signaling mediated through Src activity, leading to integrin activation. Therefore, OPN can promote integrin activation via both inside-out (by OPN/CD44V6 association) and outside-in (by OPN/RGD integrin association) mechanisms. The CD44α-mediated integrin activation was important to support OPN-mediated survival. On the contrary, the CD44-independent integrin activation is dispensable in this process.

OPN-mediated integrin activation promotes cell adhesion and matrix survival signal. Signaling events controlled by integrin regulate important biological processes, including cell adhesion. We next investigated the effect of OPN-elicted integrin activation on cell adhesion. As shown in Fig. 6A, OPN significantly enhanced the adherence of HT29 and AZ521 cells that expressed CD44 V6- or V7-containing isofoms to fibronectin. In comparison, cell attachment to poly-D-lysine or BSA was not altered by OPN treatment. Pretreatment of CD44V6-expressing AZ521 cells with blocking antibody against CD44V6 significantly suppressed OPN-elicted cell adhesion to fibronectin (Fig. 6B). In addition to the CD44-dependent effect, CD44-independent (RGD-dependent) effect was also tested by preincubating cells with anti-integrin blocking antibody on the attachment to fibronectin. As shown in Fig. 6B, anti-integrin antibody not only neutralized the integrins activated as the result of OPN-CD44 interaction but also blocked the interaction between OPN and RGD integrins, as evidenced that OPN-elicted cell adherence to fibronectin in AZ521/CD44V6 cells was completely suppressed by the anti-β1 integrin blocking antibody (P4C10) to the level observed in the control cells.

Many types of cells undergo apoptotic death when they are deprived of matrix survival signals (37). Therefore, integrin-mediated ECM signaling can support survival function to protect cells under environmental stress. We next examined whether OPN-elicted antiapoptotic function is propagated from matrix survival signal transduced by integrin. As shown in Fig. 6C, OPN-mediated survival was completely blocked by incubation of cells with anti-β1 integrin blocking antibody (P4C10), suggesting an essential role of integrin activity. Therefore, we examined whether OPN-mediated survival is derived from ECM by plating cells onto fibronectin or poly-D-lysine. As shown in Fig. 6D, plating on fibronectin supported OPN-mediated antiapoptosis in AZ521/CD44V6 cells. On the contrary, OPN did not provide any survival advantage in cells plated on poly-D-lysine. FAK is known to transduce integrinmediated signal derived from ECM. Here, we showed that blockage of matrix-derived signals by transfection and expression of FRNK completely suppressed CD44-mediated antiapoptotic response. Similarly, transfection and expression of the dominant-negative FAK (Y397F) also suppressed CD44-mediated antiapoptotic response in AZ521/CD44V6 cells (data not shown). Taken together,
the OPN/CD44-elicited antiapoptotic function is mediated through integrin activation of matrix survival signal.

Discussion
In this study, we show that both OPN and its major cell surface receptor CD44 are frequently overexpressed in human gastric cancer and that OPN confers cells an increased survival dependent on the expression of specific CD44 variant isoforms. The major finding of this study is that ligation of CD44V by OPN induces an inside-out signaling transduced through Src, leading to integrin activation, which in turn facilitates cell adhesion and enhances matrix survival signal. Several lines of evidence show that the OPN-elicited survival is derived from ECM. First, OPN treatment confers cells increased survival when cells are allowed to attach to proper ECM (e.g., fibronectin). Second, the introduction of a dominant-negative FAK (including FRNK and FAK-Y397F) completely abolished OPN-elicited survival, suggesting that FAK activation derived from integrin-ECM interaction is crucial for this process. Finally, preincubation of cells with anti-integrin β1 antibody to block integrin-ECM interaction abolished OPN-mediated survival. These data show OPN as a survival factor for cells expressing CD44V, suggesting a critical role of OPN-CD44V interaction in the tumorigenic response.

Integrin signaling is bidirectional. To examine the signaling pathway downstream OPN-CD44 interaction that leads to the inside-out integrin activation, we tested the involvement of Src kinase, PKC, PI3K, MEK, and ERK pathways. Our data showed that PP2, a Src family kinase inhibitor, but not the inhibitors specific to PI3K, MAPK, PKC, or NF-κB, blocked OPN-mediated survival. Unlike MEK or PKC inhibition, Src inhibition by PP2 also prevented pressure-stimulated adhesion. PP2 also inhibited FAK phosphorylation at Tyr397, presumably by preventing Src from phosphorylating FAK at this site (33). We further investigated the function of Src in the OPN-induced integrin activation. Binding of OPN to either the endogenously (HT29 cells) or ectopically expressed CD44V isoforms (AZ521 cells) efficiently promoted integrin activation, which was accompanied by the increase in tyrosine phosphorylation of the cytoskeletal protein cortactin (38).
with Src recruitment to CD44 and stimulation of Src kinase activity (data not shown). More importantly, OPN(RGE), unable to activate Src via an integrin, exhibited similar effect to induce integrin activation. In agreement, the dominant-negative Src (K297D)-transfected cells showed significantly reduced integrin activation induced by OPN.

Our study shows that a survival signal is induced on the ligation of CD44 by recombinant OPN at a concentration that exceeds the normal physiologic condition. Although the effective OPN concentrations within tumor cells or their surrounding stroma tissues have not been determined, increased expression of OPN is associated with tumor malignancies in several types of cancers and OPN is being investigated as a biomarker (10, 39–41). OPN concentrations within tumor cells or their surrounding stroma tissues have not been determined, increased expression of OPN is associated with tumor malignancies in several types of cancers and OPN is being investigated as a biomarker (10, 39–41).

References

Figure 6. OPN/CD44v-elicited cell survival is derived from ECM. A, OPN promotes cell adhesion to fibronectin. Subconfluent HT29, AZ521/Mock, and AZ521/CD44v6 cells were incubated with or without OPN (10 μg/mL) for 1 h at 37°C, replated on fibronectin (FN), poly-γ-lysine (PDL), or BSA, and allowed to adhere for 30 min. B, OPN-elicited cell adhesion is suppressed by antibodies against CD44v6 and integrins (5 μg/mL). AZ521/CD44v6 cells were trypsinized and pretreated with blocking antibodies as indicated for 1 h at 37°C before the incubation with OPN. Cell adhesion to fibronectin was determined as described in Materials and Methods. C, anti-integrin β3, blocking antibody blocks OPN/CD44v-mediated cell survival. Subconfluent AZ521/Mock and AZ521/CD44 clones were pretreated with or without OPN for 1 h at 37°C and further incubated in the same medium supplemented with or without OPN. Cells were UV irradiated and subjected to apoptosis assay as described above. D, fibronectin supports OPN-mediated antiapoptosis, which is blocked by the expression of the dominant-negative FAK. Subconfluent AZ521/CD44v6 cells were transfected with a control plasmid or plasmid encoding FRNK, cultured in serum-free medium for 24 h, and replated on fibronectin or poly-γ-lysine in the presence of medium with or without OPN (10 μg/mL) for 4 h. The cells were then subjected to UV irradiation followed by apoptosis assay as described above. Columns, mean of three separate experiments; bars, SD. **, P < 0.01, Student’s t test.
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