Osteopontin Prevents Curcumin-Induced Apoptosis and Promotes Survival Through Akt Activation via $\alpha_v\beta_3$ Integrins in Human Gastric Cancer Cells

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Osteopontin (OPN) is a secreted, integrin-binding matrix phosphorylated glycoprotein that is overexpressed in many advanced cancers. However, the functional mechanisms by which OPN contributes to gastric cancer development are poorly understood. Here, we report that curcumin inhibited the growth of SGC7901 cell and induced apoptosis in a concentration- and time-dependent manner, while the acquired expression of OPN in SGC7901 cells dramatically promoted cell survival under serum depletion and prevented curcumin-induced apoptosis. Furthermore, PI3-K inhibitor LY294002 attenuated OPN-mediated Akt activation. Moreover, inhibiting the binding of OPN to $\alpha_v\beta_3$ integrins reduced activation of Akt. Taken together, these results demonstrate that the pro-survival and anti-apoptosis activities of OPN in gastric cancer cells are mediated in part through PI3-K/Akt pathway via $\alpha_v\beta_3$ integrins.
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Key words: osteopontin (OPN); gastric cancer; curcumin; apoptosis; Akt

Introduction

Nonstructural extracellular matrix proteins, such as periostin (1), secreted protein acidic, rich in cysteine (SPARC) (2), and osteopontin (OPN) (3) have been found to play important roles in promoting growth, survival, invasion, angiogenesis, or in preventing apoptosis *in vitro* and *in vivo*. Recently, considerable attentions have been given to the role of OPN in the tumor progression. OPN, also known as Early T-cell activation-1 (Eta-1), is a secreted, integrin-binding matrix phosphorylated glycoprotein. OPN was originally discovered as a constituent for bone formation, but also found its expressions in macrophages, vascular smooth muscle cells, epithelial cells and endothelial cells (3–5). OPN protein contains an Arg-Gly-Asp (RGD) sequence that can bind cell surface integrins, such as $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$ (6, 7). OPN also can bind cell surface receptors including CD44 (8). Secretion of OPN and expression of CD44 may cause migration of tumor cells to specific sites of metastasis formation (9). Recent studies have implied that the expression of OPN plays an important role in tumor progression. However, the mechanisms by which OPN contributes to tumor development remain unclear. Potential mechanisms of OPN-mediated malignancy include the induction of proteases, such as urokinase-type plasminogen activator (10), as well as growth factor receptors, such as hepatocyte growth factor receptor (Met) (11) and epidermal growth factor receptor (EGFR), which are responsible for tumor motility, invasion, and metastasis (12). In addition, OPN functions as a cell survival factor, and may protect cells from undergoing apoptosis. For example, in the OPN knock-out mouse model, the apoptotic level of tubular cell has been shown to be increased, compared to wild-type mice, indicating that OPN is capable of providing survival signals to tubular epithelial cells *in vivo* (13).

Cell fate is largely dependent upon extracellular signals to activate the signal pathway of cell survival. Several signaling molecules, especially nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3-K) and Akt, play important roles in

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modulating OPN-mediated cell survival, migration and angiogenesis (14–16). Akt is one of the downstream targets of PI3-K. Upon activation of PI3-K by extracellular stimuli, Akt translocates from cytosol to the plasma membrane where it is phosphorylated at threonine 308 and serine 473, resulting in its full activation (17). OPN has been shown to mediate various tumors' survival through PI3-K/Akt signaling pathway (12, 15, 16).

Gastric cancer is the second leading cause of cancer-related deaths around the world (18). Recent studies have demonstrated that OPN highly expressed in gastric cancer tissues compared with their surrounding gastric mucosa tissues (19, 20), while the molecular mechanism of gastric carcinogenesis is not well understood because of lack of sufficient information on genetic alterations. Curcumin is the major yellow pigment extracted from turmeric, which is derived from the herb *Curcuma longa* Linn (21). Several reports have indicated curcumin plays a critical role in inhibiting proliferation and inducing apoptosis in various cancer cells including some gastric and colon cancer cells (22). Moreover, there have been reports that curcumin suppressed the OPN-induced cell migration, ECM-invasion *in vitro*, and also inhibited the OPN-induced tumor growth of nude mice *in vivo* (14). However, the molecular mechanism by which curcumin suppressed cancer survival is not well understood, and the mechanism of OPN governing the gastric cancer survival under curcumin-mediated stress has yet to be elucidated.

To investigate the molecular mechanism by which OPN promotes gastric cancer cells survival, we evaluated the effects and mechanisms of OPN on human gastric cancer cells *in vitro*. The results presented here demonstrated that OPN could promote the survival of gastric cancer cells under stressed conditions through the Akt activation via $\alpha_v\beta_3$ integrins.

Materials and Methods

Materials. Human recombinant OPN protein and rabbit anti-phospho-Akt (ser473) antibody were purchased from R&D System Europe (Abingdon, UK). The PI3-K inhibitor LY294002 was bought from Calbiochem (San Diego, CA). Mouse anti-Flag, anti-Akt, anti-β-actin, Goat anti-OPN antibodies and curcumin were purchased from Sigma Chemical Co. (St. Louis, MO). Mouse anti-$\alpha_v\beta_3$ antibody was purchased from Chemicon Inc. (LM609; Temecula, CA). All other antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA).

Generation of OPN-Producing Gastric Cancer Cells. The full open reading frame of human OPN cDNA (a kind gift from Dr. Xiao-Fan Wang Lab at Duke University Medical Center) was cloned into pcDNA3.1 mammalian expression vector (Invitrogen) with a Flag tag at C-terminus of OPN protein. The OPN/pcDNA3.1 plasmid or the empty vector was introduced into SGC7901 gastric cancer cells respectively, and the stable cell clones were obtained by 200 μg/ml hygromycin B selection in the culture medium. Three OPN-expressing clones (No. O3, O16 and O19) and one control clone (V) were chosen for the subsequent experiments.

Cell Culture and Cell Viability Assay. The human gastric cancer cell line SGC7901 was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), and was cultured as previously described (23). Cell viability was assessed by MTT assay (24). Briefly, cells were plated in 96-well plate at a density of 5×10^3 cells/well. After 24 h, cells were treated with free-serum or curcumin (0–50 μM). After the treatment, medium were removed and MTT was added to each well. After 4 h incubation at 37 °C, the MTT was discarded followed by addition of extraction buffer (90% DMSO, 10% 0.1 M Glycine-NaOH, pH10) to each well and vibration for 30 min. The optical densities (A) at 570 nm were measured using an ELISA plate reader with the extraction buffer used as a blank. The cell proliferative rate was calculated as follows: A of experimental group/A of control group $\times$ 100%. Data are reported as the means $\pm$ SD of three separate experiments.

Cell Morphological Analysis. Cells were treated with 30 μM curcumin for 48 h, the cells were fixed, and then the morphological changes were observed under a phase-contrast microscope with a CCD camera (Leica DMIRB). Meanwhile, some cells were incubated with Hoechst 33258 (Calbiochem, San Diego, CA). In addition, cells treated for indicated durations, both adherent cells and floating cells were centrifuged, and the pellet was resuspended with acridine orange and ethidium bromide; cell morphology was observed under fluorescence microscope (Leica DMIRB).

Detection of Apoptosis by Flow Cytometry. Apoptosis was identified by flow cytometry analysis as previously described (25). Briefly, cells were treated with 30 μM curcumin for 48 h, the curcumin-treated and untreated cells were harvested, washed twice with PBS and fixed in 70% ethanol at 4°C overnight. The cell pellets were suspended in propidium iodide staining solution (20 μg/ml propidium iodide and 0.2 mg/ml RNase in PBS) and incubated for 30 min at 37°C. Samples were analyzed by flow cytometry (FACS Calibur; Becton-Dickinson, Mountain View, CA). Apoptosis was measured as the percentage of cells with a DNA content lower than that of cells in G_0–G_1 in the propidium iodide intensity-area histogram plot.

Cell Survival Assay Under Curcumin Treatment. OPN-producing SGC7901 cells and the vector-transfected SGC7901 cells were incubated with 30 μM curcumin. After 48 h exposure, the cells were observed under a phase-contrast microscope and the cell numbers were determined after Trypan blue staining of viable cells in parallel plates. The experiment was repeated at least three times for each cell line. Triplicate assays were performed for each group of cells under curcumin-treated conditions and data are expressed as mean $\pm$ SD.

Western Blot Analysis. Western blot was performed as in our previous publications (24, 25). After

harvested, the experimental cells were washed and lysed. The total protein, as determined by Bio-Rad protein assay, was mixed with 4× loading buffer, and pre-heated at 95°C for 10 min. The samples were then loaded on SDS-polyacrylamine gel. The proteins were transferred onto PVDF membrane by semi-dry transfer system (Bio-Rad). The membrane was blocked in 5% milk, and then incubated 1 h or overnight at 4°C with primary antibody. After hybridization with primary antibody, the membrane was washed and incubated with HRP-labeled secondary. Final detection was performed with ECL™ (enhanced chemiluminescence) Western blotting reagents (Amersham, Pharmacia Biotech). The blot was then stripped in buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 200 mM 2-mercaptoethanol) at 50°C for 30 min. After extensive washing, the blot was used again for probing the next molecule, beginning from the blocking.

Statistical Analysis. The results of the experimental studies are expressed as mean ± SD. Statistical differences were analyzed by Student's *t* test using SPSS 10.0 software. $P < 0.05$ was regarded as significant.

Results

Overexpression of OPN in Gastric Cancer Cells Exhibits Survival Advantages in Serum-Free Condition. Previous studies have showed that the level of OPN in the serum of gastric cancer patients was elevated compared with the control (19, 20). To further assess whether stable overexpression of OPN in human gastric cancer cells could alter cell survival *in vitro*, we selected a human gastric cancer cell line SGC7901, which expresses a low level of endogenous OPN (Fig. 1A). C-terminally Flag-tagged OPN and vector constructs were transfected into the SGC7901 cells respectively, and after drug selection, three stable clones (No. O3, O16 and O19) and one vector-transfected control (V) were isolated and verified with specific antibodies against Flag-tag and OPN (Fig. 1A). Overexpression of OPN in SGC7901 cells led to a morphological change with an appearance of fibroblast-like cell shape (Fig. 1B), while it did not result in an alteration in the rate of cell proliferation in normal serum conditions *in vitro*, as measured by MTT assay (data not shown). However, under the serum-free conditions, OPN-producing cells were indeed more resistant to serum starvation in comparison with control cells ($P < 0.05$) (Fig. 1C).

Curcumin Induces Apoptosis in Gastric Cancer Cells. Cell viability was examined under different concentrations of curcumin on SGC7901 cells for 12, 24, 48 and 72 h by MTT assay. As shown in Figure 2A, curcumin inhibited the growth in a concentration- and a time-dependent manner. In comparison with the untreated cells, significant loss of viability was remarkably detected after 48 h of curcumin treatment with 30 and 50 μM ($P < 0.05$). The growth inhibitory rates of 30 μM curcumin on SGC-7901 cells at 48 and 72 h decreased quickly. Furthermore, cell morphology changes were examined after curcumin exposure. As shown in Figure 2B, 48 h after exposure to 30 μM curcumin, SGC7901 cells were shown shrinkable, rounding and fragmentary, thus taking on the typical appearance of apoptotic cells when compared to the untreated cells. We also analyzed cell morphological changes by AO/EB (Fig. 2C) and Hoechst 33258 (Fig. 2D) staining. The curcumin-treated cells also exhibited morphological changes which indicated the presence of apoptosis, including chromatin condensation and nuclear fragmentation.

Overexpression of OPN in Gastric Cancer Cells Prevents Curcumin-Induced Apoptosis. Next, we examined the biological effects of OPN on gastric cancer cells *in vitro*. 30 μM curcumin could induce significant apoptosis in SGC7901 cells (Table 1). After curcumin treatments, apoptosis was measured by means of the percentage of cells with a content of DNA lower than G_0/G_1 by analysis of the cell cycle by flow cytometry. As shown in Figure 3A, results from three independent experiments with three clones (No. O3, O16 and O19) revealed that OPN-producing SGC7901 cells were indeed more resistant to curcumin-induced apoptosis in comparison with the control cells (V) ($P < 0.01$). Similarly, the exogenous human recombinant OPN was also shown to prevent curcumin-induced apoptosis for gastric cancer cells under curcumin treatment (Fig. 3B). The increase in cell number associated with OPN expression may be due to increased cellular proliferation or resistance to cell death under stressed conditions.

OPN Activates Akt Survival Pathway Under Conditions of Growth Stresses. To further explore the molecular mechanisms by which OPN promotes cellular survival under curcumin treatment or serum depletion, we examined OPN on the activity of PI3-K/Akt cellular survival pathway by detecting the specific phosphorylation of Akt on Ser 473 under the stressed conditions with respect to growth. As shown in Figure 4A, the presence of Ser 473 phosphorylation indicates the activation of Akt pathway was readily detected in the OPN-producing SGC7901 cells under the treatment of curcumin.

Although gastric cancer cells constitutively express OPN which in turn stimulates Akt phosphorylation, we need to define whether the impact of OPN on Akt is a primary or a secondary effect to other cellular effects of long-term OPN expression. Therefore, we examined the effect of exogenous human OPN on Akt phosphorylation of the parental gastric SGC7901 cells. OPN induced a rapid Ser 473 phosphorylation of Akt under the serum-free conditions (Fig. 4B and 4C). These results suggest that OPN overexpression or introduction of exogenous human recombinant OPN in SGC7901 cells could lead to Akt activation under curcumin treatment and serum depletion conditions.

OPN Activates PI3-K/Akt Survival Pathway via the $\alpha_V\beta_3$ Integrins. PI3-K and Akt have been strongly linked to cell survival and resistance to apoptotic stimuli (17). To further confirm that OPN activates the Akt kinase

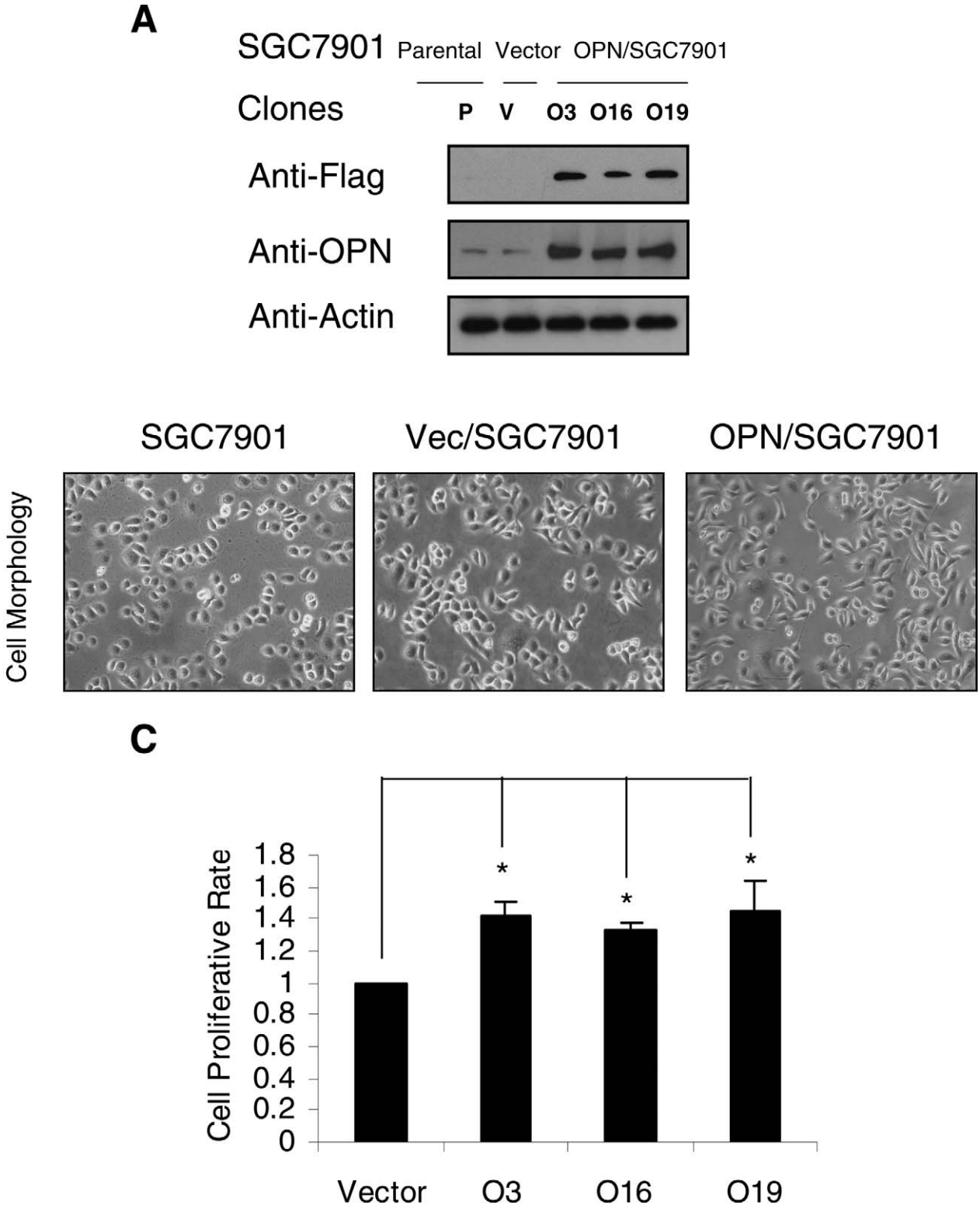

Figure 1. Characterization of OPN-overexpressing SGC7901 clones and vector control clones. (A) Equivalent number of parental SGC7901 cells (P) (2×10^6), vector control SGC7901 clone (V) and three OPN-producing SGC7901 clones (No. O3, O19, O16) were seeded in culture medium in 100 mm culture dishes. 24 h later, cells were washed with PBS and underwent starvation 4 ml serum-free medium for another 48 h. Then the conditional media from the culture were subjected to western blot assay using anti-Flag and anti-OPN antibodies. The whole-cell lysates from all samples were probed with anti-β-actin for loading control. (B) Morphology of OPN/SGC7901 cells. Magnification, ×100. (C) Effects of serum starvation on OPN/SGC7901 cell viability. Survival curves of cells after exposure to serum depletion conditions for 7 d. The viable cells were quantified by MTT assay. The cell proliferative rate was performed as described in Materials and Methods. Each point represents the mean ± SD. * $P < 0.05$ compared with vector control (SPSS 10.0).

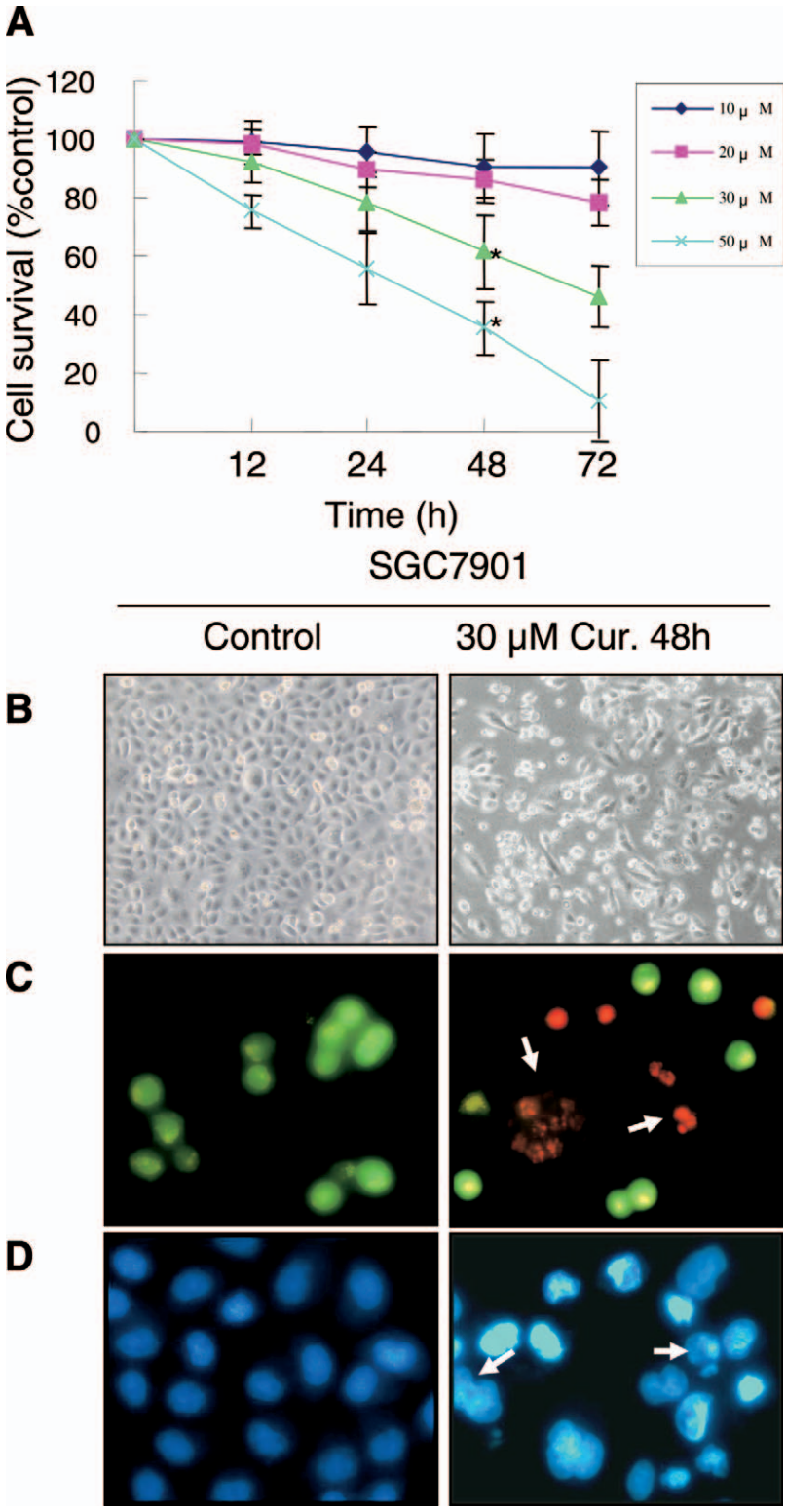

Figure 2. Effect of curcumin on the growth and morphology of gastric cancer cells. (A) Effect of curcumin on SGC7901 cell viability. The cell viability was determined by MTT assay. The data shown are means ± SD of three separate experiments done in duplicate. * $P < 0.05$ compared with untreated cells (SPSS 10.0). (B) The morphology of SGC7901 cells and the cells treated with 30 μM curcumin for 48 h. The images were captured by phase contrast microscope. Magnification, ×100. (C) Cells were fixed and stained with Hoechst 33258, and the images were captured by fluorescence microscope (Leica DM IRB). Magnification, ×200. (D) Cells were harvested and stained with AO/EB, and the images were captured by fluorescence microscope. Magnification, ×200. The arrows indicate cells undergoing apoptosis. A color version of this figure is available in the online journal.

Table 1. The Apoptotic Rate of Vector Control and OPN-Producing SGC7901 Cells Under the Curcumin Treatment

Conditions	Vector/SGC7901	OPN/SGC7901	*P* value
Serum control (2d)	0.87 ± 0.31	0.86 ± 0.19	$P > 0.05$
Curcumin (30 μM, 2d)	12.83 ± 1.20	6.19 ± 0.59	$P < 0.05$
Curcumin (30 μM, 3d)	33.45 ± 2.98	21.79 ± 2.04	$P < 0.05$

for promoting cellular survival, we used PI3-K inhibitor to block the PI3-K function and examined the effect on the OPN-mediated enhancement in cellular survival under serum-free conditions. Parental SGC7901 cells were pre-incubated with the PI3-K inhibitor, LY294002, and treated with exogenous OPN. As shown in Figure 5A, the PI3-K inhibitor lowered the impact of OPN treatment on Akt phosphorylation. These data firmly establish that PI3-K and Akt activity are required for the contribution of OPN to improve cellular survival under serum-free condition.

Previous reports indicated that OPN could bind to the $\alpha_v\beta_3$ integrins in some cancer cells (6). To determine whether the $\alpha_v\beta_3$ integrins are involved in the mediation of OPN action in gastric cancer, we pre-incubated the SGC7901 cells with antibody against the $\alpha_v\beta_3$ integrins and measured the phosphorylation of Akt on Ser473 induced by the presence of OPN. As shown in Figure 5B, the results indicate that activation of the Akt survival pathway by OPN is mediated primarily through the $\alpha_v\beta_3$ integrins signaling pathway.

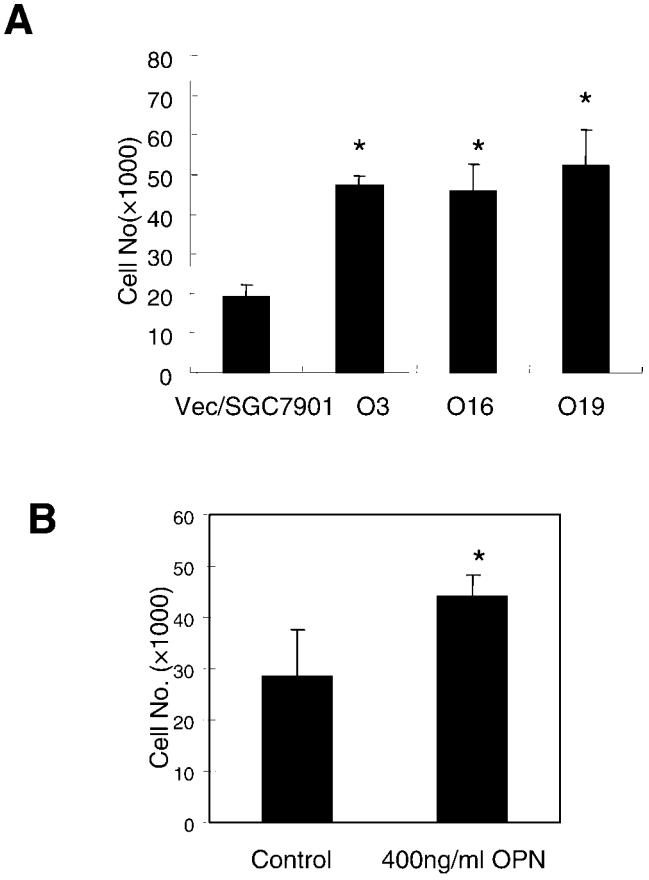

Figure 3. Overexpression of OPN in gastric cancer cells displays resistance to curcumin-induced apoptosis. (A) Cells with OPN transfection were observed, and the cell numbers were counted after trypan blue staining of viable cells. (B) Cells with exogenous human recombinant OPN treatment were observed and the cell numbers were counted after trypan blue staining of viable cells. Results in (A) and (B) were obtained from three independent experiments. * $P < 0.01$ compared with vector control (SPSS 10.0).

Discussion

OPN, an extracellular matrix protein, plays a significant role in cell adhesion, migration and metastasis (3–5). Recent studies implicated OPN as an important survival factor of various cancer cells, including gastric cancer, which may protect cells from undergoing apoptosis after a wide range of stresses *in vivo* or *in vitro* (13, 26). In this study, we observed that the acquired expression of OPN by SGC7901 cells dramatically promoted survival under serum depletion and prevented curcumin-induced apoptosis. The anti-gastric cancer properties of curcumin *in vitro* have been demonstrated by the inhibition of tumor initiation or tumor promotion (22). Here, we verified that curcumin inhibited the SGC7901 cells growth and induced apoptosis in a concentration- and time-dependent manner. Based on this observation, we found that OPN was capable to decrease the growth-inhibitory effect of curcumin-treated gastric cancer cells. Withdrawal of mitogens, growth factors, and other trophic factors by serum-starvation, serves as a potent stimulus and a driving force activating different survival mechanisms that eventually lead to apoptotic cell-death in cancer cells. In this report, we also found that OPN increased proliferation and survival of gastric cancer SGC7901 cells under prolonged serum starvation in a dose and time dependent manner. These data indicate that OPN prevents curcumin-induced apoptosis and promotes cells survival under serum depletion in human gastric cancer SGC7901 cells. The findings showed that OPN has a survival promoting activity which can further help us to interpret why a large variety of malignant cells have evolved to produce an increased level of OPN expression and have a growth advantage *in vitro*.

The PI3-K/Akt signaling pathway plays an important role in the survival response induced by a variety of growth factors, matrix adhesion and oncogene transformation (17). PI3-K/Akt pathway has been shown to mediate osteoclast activation after binding with OPN (15, 16). OPN has been shown to prevent apoptosis and confer IL-3-mediated

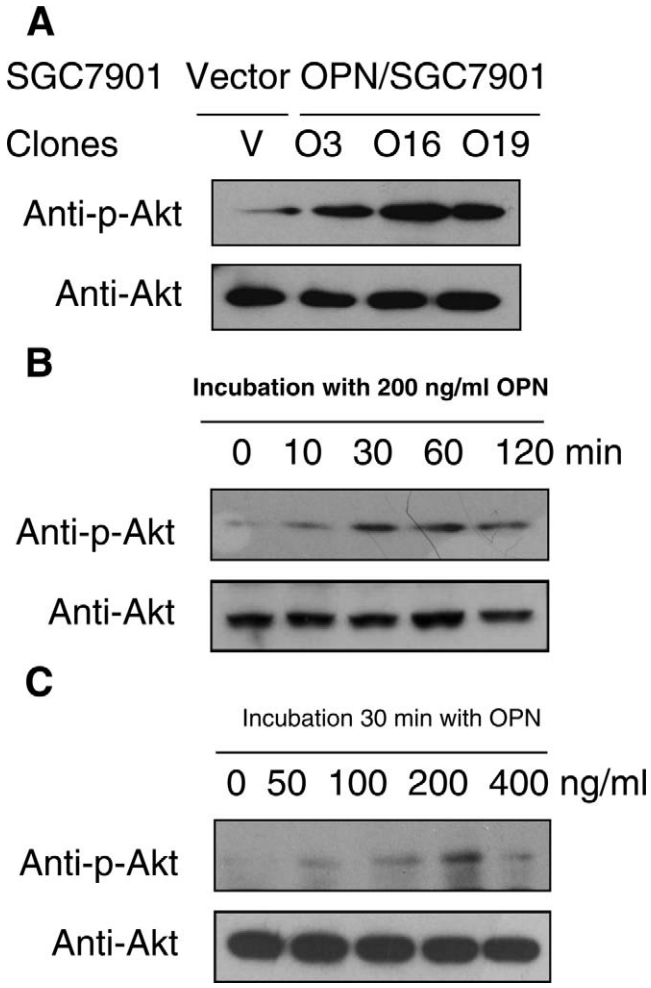

Figure 4. OPN promotes SGC7901 cells survival through PI3-K/Akt pathway activation. (A) OPN enhanced the phosphorylation of Akt on Ser 473 of gastric cancer cells under curcumin-induced stress. The vector control and OPN-producing SGC7901 cells were incubated in 30 μM curcumin for 24 h, and the whole-cell lysates from all samples were probed with anti-p-Akt. (B) The parental SGC7901 cells were serum-starved overnight, and treated with 200 ng/ml human OPN (hOPN) for 0, 10, 30, 60, 120 min before the detection of the phosphorylation of Akt on Ser 473. (C) The parental SGC7901 cells were serum-starved overnight, and treated with 0, 50, 100, 200, 400 ng/ml human OPN (hOPN) for 30 min before the detection of the phosphorylation of Akt on Ser 473. All blots in (A), (B) and (C) were stripped and reblotted with Akt antibody for the loading control. This experiment was repeated at least three times independently and similar results were obtained.

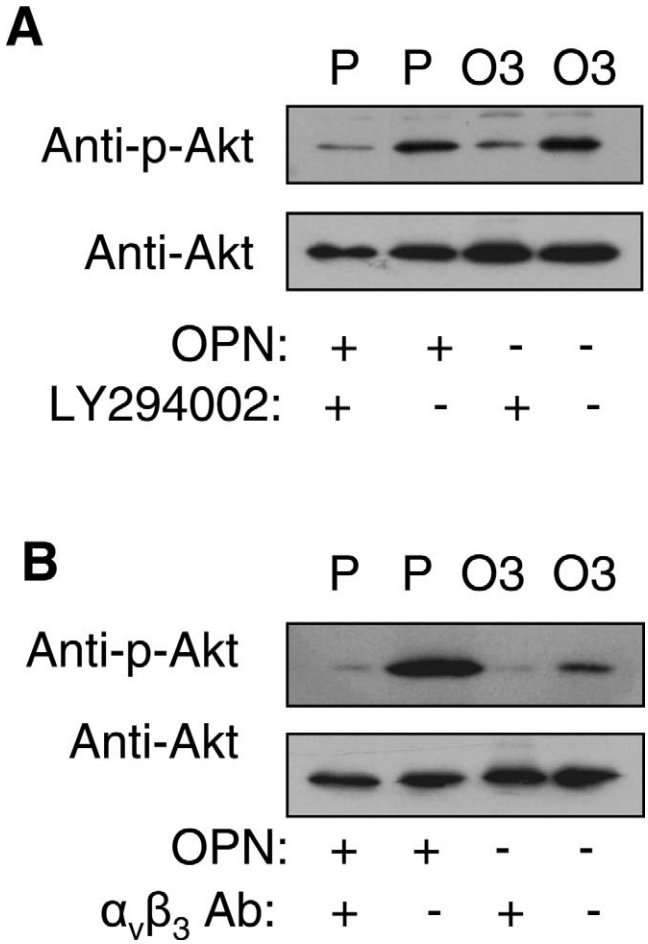

Figure 5. OPN activates PI3-K/Akt pathway via the $\alpha_v\beta_3$ integrins to enhance cellular survival. (A) PI3-K activity is necessary for OPN-mediated Akt phosphorylation on Ser 473. 24 h serum-starved parental SGC7901 cells were pretreated for 3 h with LY294002 (50 μM), followed by treatment with PBS or human OPN (200 ng/ml) for 30 min and OPN-producing SGC7901 cells (O3) were also treated with LY294002 (50 μM) for 3 h, and lysed for detection of the phosphorylation of Akt on Ser 473. Blots were stripped and reblotted with Akt antibody. (B) The phosphorylation of Akt on Ser 473 was blocked by the presence of an antibody against the $\alpha_v\beta_3$ integrins. 24 h serum-starved parental SGC7901 cells were pretreated with 10 μg/ml anti-$\alpha_v\beta_3$ mAb for 12 h, followed by treatment with PBS or human OPN (200 ng/ml) for 30 min, and lysed for detection of the phosphorylation of Akt on Ser 473. Blots were stripped and reblotted with Akt antibody. All of these experiments were repeated three times independently and similar results were obtained.

survival advantage through PI3-K/Akt pathway activation in murine B cells (27). Here, we were also interested in examining the level of PI3-K/Akt activity during curcumin treatment. In our experiments, we found that OPN upregulated the phosphorylation of Akt on Ser 473 in the SGC7901 cells under curcumin treatment or serum depletion. This effect was substantially inhibited by LY294002, an inhibitor of the upstream Akt effector, PI3-K. These data clearly indicated that OPN-inhibition of the apoptogenic activity of curcumin is at least partially dependent on the upstream Akt effector, PI3-K. The above results suggest that the two closely inter-connected cell survival/apoptotic pathways (PI3-K/Akt) activated by OPN might potentially synergize and provide a growth and survival advantage to gastric cancer cells.

OPN binding has been found to involve both integrins and nonintegrin receptors in different cell types and interference in their interaction may profoundly curtail the dissemination of various cancers. Previous studies indicated that OPN binds to a variety of cell surface receptors including integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and CD44 (6–8). OPN functions are primarily executed through a division of labor between integrin $\alpha_v\beta_3$ and CD44 (28–30). Recently,

correlative studies suggest that $\alpha_v\beta_3$ may be particularly associated with increased tumor aggressiveness (31, 32). However, the relative contribution of these integrins to gastric cancer cell malignancy remains uncertain. To gain further insight into OPN's mechanism of action in gastric cancer SGC7901 cell survival response, we pre-incubated the SGC7901 cell with antibody against the $\alpha_v\beta_3$ integrins and measured the phosphorylation of Akt on Ser 473 induced by the presence of OPN. The results demonstrated that, via binding to the $\alpha_v\beta_3$ receptor, OPN cannot activate the PI3-K/Akt kinase. The *in vitro* model system described here indicates that the $\alpha_v\beta_3$ integrins may be important for gastric cancer cell response to both exogenously and endogenously produced OPN.

In summary, we have demonstrated that OPN promotes gastric cancer SGC7901 cells survival in the face of cellular stresses induced by serum withdrawal or curcumin treatment. The apoptotic rate of OPN-overexpressing gastric cancer cell lines is reduced relative to control cells and is associated with activation of the anti-apoptotic kinase, Akt. As expected, the impact of OPN on Akt activation is dependent on PI3-K activity as measured by the ability of PI3-K antagonists to block Akt phosphorylation and increased survival imparted by OPN. Our findings also suggest that the binding of OPN to the $\alpha_v\beta_3$ integrins activates the pro-survival PI3-K/Akt signal pathway and protects SGC7901 cells from undergoing apoptosis. The elucidation of these intricate mechanisms of OPN targets to the development of a possible therapeutic option that combines cytotoxic therapies to treat gastric cancer.

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