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Beclin1-induced Autophagy Abrogates Radioresistance of Lung Cancer Cells by Suppressing Osteopontin

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Osteopontin(OPN)/Beclin-1(BECN1)/Gamma-irradiation/p53/Angiogenesis.

Osteopontin (OPN) serves as an indicator of resistance to radiotherapy. However, the role of OPN in the development of acquired radioresistance in human lung cancer cells has not yet been fully elucidated. Therefore, the potential importance of OPN as a marker of lung cancer with a potential significant role in the development of radioresistance against repeated radiotherapy has prompted us to define the pathways by which OPN regulates lung cancer cell growth. In addition, autophagy has been reported to play a key role in the radiosensitization of cancer cells. Here, we report that increased OPN expression through induction of nuclear p53 following irradiation was inhibited by exogenous beclin-1 (BECN1). Our results clearly show that BECN1 gene expression led to induction of autophagy and inhibition of cancer cell growth and angiogenesis. Our results suggest that the induction of autophagy abrogated the radioresistance of the cancer cells. Interestingly, we showed that knockdown of OPN by lentivirus-mediated shRNA induced the autophagy of human lung cancer cell. Taken together, these results suggest that OPN and BECN1 can be molecular targets for overcoming radioresistance by controlling autophagy.

INTRODUCTION

Osteopontin (OPN) is a secreted, integrin-binding, glycosylated phosphoprotein expressed in most cell types.1,2 Elevated OPN expression has been observed in a variety of human tumors, including breast,3 prostate,4 colon,5 ovarian,6 and stomach cancers.7 The overexpression of OPN has also been frequently detected in lung cancer patients such that elevated serum levels of OPN are associated with lung cancer status8,9 and poor survival.10,11 OPN has also been demonstrated to play a key role in angiogenesis.12,13 A recent study suggested that OPN may serve as an indicator of tumor re-growth in response to radiotherapy.14 Together, the above results suggest that targeting OPN may provide a rational approach to enhancing lung cancer therapy.

Autophagy is a catabolic process involving the degradation of cytoplasmic proteins and organelles through the lysosomal machinery. Autophagy has been implicated in physiological cell death during development15 and is reported to be involved in cancer cell death upon treatment with chemotherapeutics and irradiation.16,17 Autophagic cell death is morphologically characterized by an intact nucleus and the accumulation of cytoplasmic double-membrane autophagic vacuoles.18,19 The formation of an autophagosome is initiated by class III phosphoinositide 3-kinase (PI3K) and autophagy-related gene (Atg) 6 (also known as beclin-I, BECN1). BECN1 is one of the essential components involved in autophagosome formation, and it displays increased expression during autophagy.16,20 Microtubule-associated protein light chain 3 (LC3) is an autophagosome marker, and conversion of its cytosolic form, LC3-I, to its autophagic membrane form, LC3-II, is another key step in the induction of autophagy.21,22

Despite intense research efforts focused on the treatment...
of lung cancer, it is still the leading cause of cancer-related death worldwide. Radiation in combination with chemotherapy has long been used as a reasonably effective short-term treatment regimen for lung cancer. However, some malignancies are relatively resistant to radiation upon repeated treatment and may eventually recover self-proliferative capacity. In general, the roles of autophagy are known as a mode of radiosensitization rather than protection from radiation injury and cell death. It can therefore be argued that one of the explanations for the relatively poor long-term success rate in treatment of lung cancer may be associated with radioresistance. In fact, several lines of evidence suggest that deficits in apoptotic machinery can lead not only to abnormal proliferation but also to insensitivity to radiotherapy. Exploiting autophagy could have the capacity to improve the efficacies of current therapies.

In this study, we show that the induction of autophagy through BECN1 overexpression resulted in decreased OPN levels as well as inhibition of cell growth and angiogenesis. Taken together, our results suggest that autophagy-induced suppression of OPN may represent a method for enhancing treatment of lung cancer.

MATERIALS AND METHODS

Cell culture and irradiation

A549 cells obtained from American Type Culture Collection (Rockville, MD; CCL-185) were grown in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin (GibcoBRL; Grand Island, NY). Cells were exposed to gamma-rays from a 137Cs source at a dose rate of 5 Gy/min using cells grown to 70–80% confluency in T25 flask and transfected with 5 to 70–80% confluence in a T25 flask were transfected with 5 to 70–80% confluence in a T25 flask and transfected with

shRNA-delivered lentivirus construct for OPN knock-down

The shRNA sequence targeting human OPN mRNA was described previously. Lentivirus containing shRNA targeting OPN was generated by following the manufacturer’s instructions (BLOCK-iT™ Lentiviral RNAi Expression System, Invitrogen), after which the virus titer was determined using an HIV-1 p24 ELISA KIT (PerkinElmer Life Sciences; Boston, MA).

OPN siRNA preparation

siRNA targeting OPN was generated from siXpress Human U6 PCR Vector System by Mirus Bio Corporation according to manufactures recommendations. The OPN and scrambled siRNA sequence targeting human OPN were the same as for lentiviral targeting vector. Cells were grown to 70–80% confluency in T25 flask and transfected with 5 μg of plasmid using TransIT™-LT1 reagent. After 48 h incubation, cultured cells and media were harvested.

OPN immunoassay

Cells were irradiated and transfected with hBECN1 construct. OPN levels from cultured medium were determined by quantikine human OPN immunoassay from R&D Systems (Minneapolis, MN), and the results were read on a Microplate Reader (Bio-Rad Laboratory) at OD 450 nm.

Luciferase assay

Tp53-responsive genomic sequence (CTGCTTGCTTAGGCCAGCTC) of the mouse OPN gene (GenBank ID: D14816) was inserted into the pGL3-Promoter vector (Promega). A549 cells were grown on 6-well plates and then transfected with each reporter gene using TransIT™-LT1 reagent. After transfection, cells were irradiated with 0 or 5 Gy, and incubated for the indicated times. Slides were washed with PBS and fixed in 4% paraformaldehyde. Cells were washed and fixed again with methanol:acetone. After being blocked, cells were incubated in primary antibodies, washed, and incubated with fluorescence-conjugated secondary antibodies. Nuclei were stained with DAPI in a dark room. After washing, the slides were visualized using a fluorescent microscope (Carl Zeiss; Thornwood, NY).
FACS analyses

Single-cell suspensions of A549 cells were prepared after radiation, and cells were incubated with a mouse anti-Bax antibody in PBS–3% bovine serum albumin for 30 min at 4°C. The cells were washed twice; FITC-conjugated anti-mouse antibody or TRITC-conjugated anti-rabbit antibody was added, respectively; and the cells were incubated at 4°C for 30 min. The cells were then washed and analyzed by FACS Calibur (BD Bioscience; San Jose, CA).

Annexin V-FITC staining

Forty-eight hours after radiation, cells were processed with an EzWay Annexin V-FITC Apoptosis Detection kit (KOMABIO TECH INC., Korea) according to the manufacturer’s recommendations. Then, the samples were analyzed by FACS Calibur.

RT-PCR

A549 cells were irradiated with 0 or 5 Gy of gamma-ray and incubated for 24 h. Total RNA was isolated and subjected to RT-PCR (ONE-STEP RT-PCR PreMix kit; iNtRON Biotechnology, INC., Korea) following the manufacturer’s protocol. Two μg of RNA were amplified with specific primers at the following cycling conditions: RT step - reverse transcription reaction at 45°C for 30 min, denaturation of RNA : cDNA hybrid at 94°C for 5 min, PCR step - denaturing at 94°C for 30 sec, annealing at 53°C for 30 sec, and extension at 72°C for 1 min, 25 cycles. As an internal control, we amplified GAPDH. The sequence of the primers used for RT-PCR were as follows; for BECN1, forward 5′-GGAGAAACTCCAGCGAAGAC-3′ and reverse 5′-GTGCTTCCTGCTGTG-3′; for ATG5, forward 5′-TGGTTGACGAGATCCGCGA-3′ and reverse 5′-CCATTCCAGCTTGCTGGT-3′; for LC3, forward 5′-GAGGTACAGCAGATCCGCGA-3′ and reverse 5′-TCAGAAGCCGAAGTGTG-3′; for GAPDH, forward 5′-ACACGTCATGGGAGACGTTG-3′ and reverse 5′-GTCAGGTCCACCACACTGAC-3′. Products were analyzed by electrophoresis on 2% agarose gels.

Western blot analysis

Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in TTBS containing skim milk. Immunoblotting was performed by incubation with the corresponding primary antibodies and then with HRP-conjugated secondary antibodies. After washing, the bands-of-interest were visualized using a luminescent image analyzer LAS-3000 (Fujifilm; Tokyo, Japan). In the experiments requiring nuclear and cytoplasmic fractionation, the fractions were isolated using a Nuclear Extract Kit from Active Motif (Carlsbad, CA).

Cell proliferation assay

Cell proliferation was measured using a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories; Kumamoto, Japan). Briefly, 10 μl CCK-8 solution was added to each well to a final volume of 100 μl of culture medium containing viable cells. After additional incubation for 3 h, spectrophotometric absorbance at 450 nm was measured.

Zymography assay

Cell culture medium was concentrated using an Amicon ultra-15 centrifugal filter device (Millipore; Billerica, MA). Equal amounts of protein were separated in 10% SDS-polyacrylamide gel containing 1% gelatin. The gel was then washed two times with 2.5% Triton X-100 and subsequently incubated in a collagenase buffer. The gel was stained with Coomassie blue and destained. Negative staining showed the zones of gelatinolytic activity.

Statistical analysis

Data are expressed as means ± SEM. Student’s t test was used for comparison between two groups. All statistical analyses were performed using GraphPad Software version 4.02 (San Diego, CA). Quantification of Western blot analysis was performed using the Multi Gauge version 2.02 program (Fujifilm).

RESULTS AND DISCUSSION

Gamma-irradiation increases OPN protein levels in a p53-dependent manner

OPN can serve as a surrogate marker of tumor recovery after radiotherapy. Radiotherapy involves the fractionated delivery of high-energy x-rays, which exert a cytotoxic effect by producing free radicals within target tissues. It is known that tumor hypoxia is a major determinant of radiore sistance. Since OPN expression is increased in the presence of tumor hypoxia, elevated OPN levels can be associated with radiotherapy resistance. In addition, recent lines of evidence show that OPN knockdown causes weak radiosensitivity. Therefore, OPN seems to be an attractive target to improve the effectiveness of radiotherapy. In this study, to investigate the effect of irradiation on OPN levels, A549 cells were exposed to 0 or 5 Gy of gamma-radiation, after which OPN expression levels were measured by Western blot analysis, ELISA, and immunofluorescence analysis. The results show that radiation induced OPN expression in A549 cells (Fig. 1A, B, C).

The OPN promoter is responsive to many stimuli, including various forms of physical stress, cytokines, growth factors, and hormones through different DNA-binding sites. Recent studies have identified a functional p53-responsive element in the OPN gene promoter region. We therefore analyzed the potential role of p53 in regulating OPN in response to radiation using the consensus p53 bind-
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Fig. 1. Radiation increases OPN in a p53-dependent manner in A549 cells. (A and B) Gamma-ray irradiation increases both cellular and secreted OPN levels. Cells were irradiated with 0 or 5 Gy and incubated for 24–96 h, followed by lysis and collection. Lysates were used for Western blotting (A) and cultured media were used for ELISA assay (B). Values are the means ± SEM (n = 3 per group). *p < 0.05 vs 0 Gy-24 h, **p < 0.01 vs 0 Gy-96 h. (C) Fluorescence imaging of OPN after radiation. Irradiated cells were incubated for 24 h, followed by fixing and immunostaining for OPN (red via TRITC) and nuclei (blue via DAPI). The scale bar represents 10 μm. (D) Western blot analysis of p53 in the cytosol and nucleus. Cells were collected and then separated into cytosolic and nuclear fractions using a Nuclear Extract Kit. GAPDH and Histone H1 were used as controls for the cytosol and nucleus, respectively. (E) Luciferase assay for OPN promoter activity. Cells were transiently transfected with pGL3-OPNwt or pGL3-OPNmut vector, irradiated with 0 or 5 Gy, and then incubated for 48 h. Values are the means ± SEM (n = 5 per group). ***p < 0.001 vs OPNwt-0 Gy. (F) p53-dependent induction of OPN by radiation. Cells were transiently transfected with p53 shRNA vector, irradiated with 0 or 5 Gy, and then incubated for 24 h. Scr shRNA: scrambled shRNA. (G) Gamma-ray irradiation induces changes in levels of p53, p21, and Bcl-2. Cells were irradiated with 0 or 5 Gy and incubated for 48–72 h, after which lysed proteins were analyzed by Western blotting.

ing sequence in the mouse OPN gene. A549 cells were transiently transfected with pGL3-OPNwt/pGL3-OPNmut and irradiated with 0 or 5 Gy. Our results clearly show that 5 Gy of irradiation significantly enhanced the relative luciferase activity of pGL3-OPNwt, whereas no increase was detected with the pGL3-OPNmut (Fig. 1E). On the other hand, OPN levels were clearly increased in control cells in response to irradiation, and OPN levels remained unchanged in p53 knockdown cells (Fig. 1F). We also showed that increased OPN protein expression after gamma-irradiation might have induced nuclear p53 (Fig. 1D). These results demonstrate that irradiation increased OPN in a p53-dependent manner.

The p53 protein is a key molecule responsible for G1 phase cell cycle arrest in response to DNA damage, which results in upregulation of p21. Additionally, p53 interacts with Bcl-2 and Bcl-XL proteins and blocks their functions. Irradiation markedly increased the protein levels of p53 and p21 in A549 cells and decreased the level of Bcl-2 (Fig. 1G). Our results reveal that irradiation-induced p53 reversely inhibited Bcl-2 protein expression.
Fig. 2. Radiation induces autophagy and apoptosis at early stage. (A) Gamma-ray induces changes in levels of autophagic and apoptosis-related proteins—BECN1, ATG5, LC3, and Bax. Cells were irradiated with 0 or 5 Gy and incubated for 48–72 h, after which lysed proteins were analyzed by Western blotting. (B) Flow cytometric analysis of Bax after radiation. Cells were irradiated with 0 or 5 Gy and incubated for 48 h, and each group of cells incubated with Bax antibody and fluorescence-labeled secondary antibody, then, analyzed by flow cytometry. (C) Annexin V-FITC staining after radiation. Cells were irradiated with 0 or 5 Gy and incubated for 48 h, and each group of cells stained with Annexin V-FITC and propidium iodide (PI), then, analyzed by flow cytometry. (D) RT-PCR analysis of autophagic proteins (BECN1, ATG5, and LC3) after radiation. Cells were irradiated with 0 or 5 Gy and incubated for 24 h, and then total RNA was isolated and subjected to RT-PCR. Values are the means ± SEM of three independent experiments. *p < 0.05 and **p < 0.01 vs 0 Gy. (E) Fluorescence imaging of BECN1 and LC3 after radiation. Immunostaining was performed for BECN1 and LC3 (red via TRITC) and nuclei (blue via DAPI) after 48 h. The scale bar represents 10 μm.
**Gamma-irradiation increases autophagy and apoptosis at early stage**

A recent study reported that BrdU treatment and sequential UV irradiation induces an increase in the ratio of pro-apoptotic Bax to that of Bcl-2, resulting in DNA damage compared to irradiated, unsensitized cells. BrdU also synergizes with radiation to increase autophagic cell death, including conversion of LC3B-I into LC3B-II. We therefore investigated the effects of gamma-radiation on autophagy and apoptosis-related proteins. The Western blot result found that radiation induced levels of Bax, BECN1, ATG5, and LC3 at 48 h (Fig. 2A). The increase in apoptotic and autophagy proteins in response to radiation were confirmed by FACS analysis (Fig. 2B) and annexin V staining (Fig. 2C).

![Fig. 2A](image)

![Fig. 2B](image)

![Fig. 2C](image)

![Fig. 2D](image)

![Fig. 2E](image)

Fig. 3. Continued.
after 48 h and by RT-PCR after 24 h (Fig. 2D), respectively. Furthermore, immunofluorescence analysis of BECN1 and LC3 clearly confirmed Western blotting results (Fig. 2E). However, induction of apoptosis with decreased autophagy was observed after 72 h of irradiation (Fig. 2A). Therefore, these results suggest that radiation induced both apoptosis and autophagy at early stage while autophagy was not steadily increased.

**OPN counteracts BECN1 protein expression**

To investigate whether or not OPN regulates BECN1 expression, A549 cells were transiently transfected with OPN expression vector. Increased OPN levels correlated with a marked decrease in BECN1 protein levels after 48 h (Fig. 3A). Therefore, the reduction of BECN1 at 72 h after irradiation could have been OPN-dependent since exogenous OPN decreased BECN1 directly. We next hypothesized...
that BECN1 overexpression induced autophagy in human lung cancer cells with simultaneous suppression of lung cancer cell growth. To determine whether or not overexpression of BECN1 inhibits the recovery of cancer cell re-growth after radiation, we used BECN1 expression vector. Transient transfection of A549 cells with BECN1 resulted in autophagy, as evidenced by the conversion of LC3, increased ATG5 protein level, and suppression of cell growth (Fig. 3B, C). Densitometric analysis clearly confirmed the Western blot results (Fig. 3B, right). Moreover, we measured the level of OPN by Western blot (Fig. 3D) and ELISA assay (Fig. 3E). We determined that BECN1-treated cells significantly reduced the amount of OPN compared to irradiated and vector controls. To examine whether or not BECN1 overexpression can overcome highly expressed OPN to induce autophagy, we generated a stable cell line constitutively expressing OPN. Following hBECN1 transfection into OPN-expressing cells, OPN and Bcl-2 protein levels decreased while the pro-

![Fig. 4. BECN1 suppressed cancer cell growth and angiogenesis. (A) BECN1 suppresses the OPN expression. OPN knock-downed stable cell line by lentivirus-mediated shRNA was used as negative control. (B) BECN1 suppresses MMP-2 protein expression and activity. Culture media for A549 (Con), OPN, and BECN1 expressing cells were used for Western blot and gelatin zymography assay. Right panels represent densitometric analysis. Values are the means ± SEM of three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 vs Con (control), #p < 0.01 and ##p < 0.001 vs OPN (OPN stable cell). Con: A549 control cells, OPN: OPN-expressing cells, BECN1: BECN1-expressing cells, W.B.: Western blot, Zymo.: Zymography, Pro: Pro-MMP-2, Active: Active-MMP-2. (C–E) BECN1 suppresses VEGF and PCNA expression and inhibits cancer cell growth. A549, OPN, and BECN1-expressing cells were used for Western blot (C and D) and cell proliferation assay (n = 10 per group) (D). Cells stably expressing OPN were used as a positive control (OPN). Values are the means ± SEM of three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 vs Con (control), #p < 0.05 and ##p < 0.001 vs OPN (OPN stable cell). Con: A549 control cells, OPN: OPN-expressing cells, BECN1: BECN1-expressing cells.)
tein levels of BECN1, ATG5, and LC3 II increased (Fig. 3F). To determine the effect of OPN on BECN1 levels, A549 cells were treated with lentivirus expressing shRNA or siRNA expression vector targeting OPN. Knockdown of OPN in cells also led to an increase in the protein levels of BECN1, ATG5, and LC3 II (Fig. 3G). Together, the above results show that downregulation of OPN may have induced the autophagy of human lung cancer cells.

**Decreased OPN by BECN1 suppresses angiogenesis and cancer cell growth**

Based on our results which indicate stable expression of BECN1 in A549 cells led to decreased OPN level (Fig. 4A), we next examined whether or not stable expression of BECN1 would alter cancer cell growth and angiogenesis. Western blot, zymography, and CCK assay were carried out in stable A549 cells expressing BECN1. Stable A549 cells expressing OPN were used for induction of angiogenesis. OPN is a metastasis gene that contributes to stress-dependent angiogenesis. Recent studies reported that recombinant OPN induced the expression and activation of MMP-2 in human cancer cells. Our results clearly show that the levels of both the pro- and active- forms of MMP-2 were significantly decreased in stable cells expressing BECN1 com-

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**Fig. 5.** Induction of autophagy abrogates radioresistance of irradiated cells stably expressing BECN1. (A and B) Expression levels of OPN, BECN1, and LC3 after radiation as detected by Western blotting. Cells stably expressing BECN1 were irradiated with 0 or 5 Gy and incubated for 24–96 h. (C) A549 and cells stably expressing BECN1 were used for cell proliferation assay. Cells were irradiated with 0 or 5 Gy, and cell viability was measured using CCK-8. Values are the means ± SEM (n = 10 per group). (D) BECN1 induces p53 and p21 protein expression and inhibits apoptosis-related proteins (cleaved-PARP and Bax). A549 (control) and stably expressing BECN1 cells were used for Western blots analysis. (E) BECN1 induces autophagosomes in the cytoplasm. Immunostaining was performed for LC3 (green via FITC) and nuclei (blue via DAPI) after 48 and 72 h. The scale bar represents 10 μm.
pared to OPN-expressing cells as well as control cells. Den-
sitometric analysis also confirmed the Western blot and
zymography results (Fig. 4B). A similar pattern of expres-
sion was clearly observed for VEGF (Fig. 4C). Vascular
Endothelial Growth Factor (VEGF) has been reported to reg-
ulate angiogenesis through mechanisms involving VEGF-
induced proteins, including OPN, and it has been shown
that tumor-derived OPN contributes to tumor development
and recurrence by way of VEGF-dependent angiogenesis.

Our results show that decreased OPN by BECN1 could have
suppressed angiogenesis.

In addition, the effect of BECN1 on cell proliferation was
also investigated. BECN1 decreased the level of PCNA (cell
proliferation marker), as detected by Western blotting (Fig.
4D). To determine whether or not stable expression of
BECN1 affects cell death, CCK assay was performed to
measure cell death. The results found that stable cells
expressing OPN increased cell growth, whereas a significant
decrease in growth was observed in cells stably expressing
BECN1 (Fig. 4E). Together, our works clearly demonstrate
that overexpression of BECN1 may contribute to inhibition
of cancer cell growth and angiogenesis.

Induction of autophagy abrogates radioresistance

To evaluate whether or not induction of autophagy abro-
gates the radioresistance of cells, Western blotting and cell
proliferation assay were carried out in irradiated cells stably
expressing BECN1. Our results show that OPN protein lev-
els in stably expressing BECN1 cells treated with radiation
did not increase after 24 h and 48 h while irradiated A549
cells showed an increase in OPN levels after 48 h (Fig. 5A)
and therefore did not decrease BECN1 and LC3 II expres-
sion after 72 h (Fig. 5B). Autophagy is negatively regulated
by the mammalian target of rapamycin (mTOR) and can be
induced in all mammalian cell types by the mTOR inhibitor
rapamycin. In this study, rapamycin was used for induc-
tion of autophagy by regulating the autophagosomes formation and/
or decreasing their infusion with lysosomes. In this
study, we first report that BECN1 increases p53. BECN1
seems to inhibit lung cancer cell growth by induction of p53
through regulating autophagy. These findings combined with
increased autophagy may contribute the cell death and tumor
suppression.

Together, induction of autophagy seems to suppress tumor
formation/progression by several proposed mechanisms,
including the degradation of specific molecules/organelles
critical for cell growth, the removal of damaged organelles
which may cause toxicity, and the induction of autophagic
cell death.

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