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Enhanced Cell Killing by Overexpression of Dominant-negative Phosphatidylinositol 3-Kinase Subunit, Δp85, Following Genotoxic Stresses

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Phosphatidylinositol 3-kinase (PI3-K) is a heterodimer of a regulatory subunit, p85, and a catalytic subunit, p110. A number of previous reports showed that PI3-K functions in diverse cellular phenomena such as cell proliferation, glucose catabolism, cell adhesion, and vesicle transport. It is also well known that a survival signal from the receptor tyrosine kinases utilizes Akt via PI3-K to protect cells from apoptosis. To examine the role of PI3-K in cellular sensitivity to genotoxic stresses such as cisplatin and ultraviolet (UV), we introduced deletion type p85 (Δp85) into two human glioblastoma cell lines (T98G and A172) and one melanoma cell line (G361). The Δp85 works in a dominant-negative fashion on PI3-K activity by disrupting its p85/p110 interaction. In all three transfected cell lines, the overexpression of Δp85 rendered the cells markedly more sensitive to these DNA-damaging stresses than the cells transfected with the vector alone. Apoptosis was vigorously induced in cells overexpressing Δp85 following the treatment. The present results imply that PI3-K plays a critical role in determining cellular sensitivity to genotoxic stresses in human cancer cells, and that disruption of the p85/p110 interaction of PI3-K may be a potential molecular target for developing a novel strategy for cancer treatment.

Key words: Phosphatidylinositol 3-kinase — Dominant-negative p85 — Sensitization — Cisplatin — UV

Chemotherapy and radiotherapy have been widely used in the treatment of cancer. However, it is not possible to cure all kinds of cancer because of the presence of resistant tumor types or a resistant subpopulation in tumors to these therapies. Since chromosomal DNA is the primary target of chemotherapy and radiotherapy, cellular sensitivity toward these therapies is regulated by the DNA damage response pathways including the induction of apoptosis, cell cycle checkpoints, and repair of DNA double-strand breaks. Therefore, molecules involved in these pathways are possible targets for cancer treatment.

Phosphatidylinositol 3-kinase (PI3-K) is a heterodimer consisting of p85 regulatory subunit and p110 catalytic subunit.[1,2] PI3-K converts phosphatidylinositol (PtdIns), phosphatidylinositol (4) monophosphate (PtdIns(4)P) and PtdIns(4,5)P_2 to PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respectively. Major products of PI3-K are PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, of which the latter serves as a second messenger in diverse cellular phenomena such as mitogenesis, intracellular vesicle trafficking/secretion, and regulation of the cytoskeleton.[1] It is also known that a pathway from the receptor tyrosine kinases utilizes PI3-K to activate Akt, a kinase which exerts a protective function against apoptosis through phosphorylation of Bad.[3,4]

Several molecules are known to be involved in cellular sensitivity to genotoxic stress. Reports have demonstrated an association of molecules for cell proliferation or regulation of apoptosis with cellular sensitivity to cisplatin and ultraviolet (UV).[5–8] Although PI3-K is also involved in these cellular phenomena, the role of PI3-K in cellular sensitivity to genotoxic stress remains unclear.

To examine the role of PI3-K in sensitivity to cisplatin and UV, we introduced the well characterized deletion type p85 (widely referred to as Δp85[9–11]) into two human glioblastoma T98G and A172 cells and a human melanoma G361 cell. Δp85 lacks the binding site for the p110 catalytic subunit, and works in a dominant-negative fashion for PI3-K activity.[12] We have found that overexpression of Δp85 vigorously sensitized the human cancer cells to these stresses. Overexpression of Δp85 potentiated the induction of apoptosis following the genotoxic treatment. The present results imply that PI3-K plays a critical role in determining cellular sensitivity in human cancer cells to cisplatin and UV, and that disruption of the p85/p110 interaction may provide a novel strategy for cancer treatment.

MATERIALS AND METHODS

Chemicals and cells Human glioblastoma cell lines (T98G and A172) and human melanoma cell line (G361) were used in this study. T98G and A172 were kindly provided by Dr. Day (Cross Cancer Institute, Edmonton, AB,

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Canada). G361 cells were provided by Dr. Utsumi (Research Reactor Institute, Kyoto University, Osaka). Cells were cultured at 37°C with 95% air and 5% CO_2 in Dulbecco's modified Eagle's minimum essential medium (Nikken, Kyoto) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

Plasmids and transfection The plasmid pGEX-Δ85a (kindly provided by Dr. Kasuga, the Second Department of Internal Medicine, Kobe University School of Medicine, Kobe) encodes bovine deletion type p85a (Δp85) which lacks 35 amino acids in residues 479–513 of the binding sequence with p110 of PI3-K. The insert was liberated by digestion with *Bam*HI and *Eco*RI restriction enzymes, and was subcloned between the *Bam*HI and *Eco*RI sites of the pcDNA3 expression vector (Invitrogen, Carlsbad, CA). We employed the electroporation method for transfection and selected cells with 400 μg/ml of neomycin. Cultures were maintained until neomycin-resistant colonies appeared.

Cell survival Cell survival was assessed with a clonogenic assay. To determine cellular sensitivity to cisplatin serially-diluted cells were plated and, after 8 h of incubation, cisplatin was added to the culture medium to the indicated concentration.

To determine cellular sensitivity to UV, the plated cells were washed with phosphate-buffered saline (PBS) (−) and irradiated with a bank of two 15 W germicidal UV lamps (predominantly 254 nm; Toshiba Corp., Tokyo) at a dose rate of 1.2 J/m^2/s. After irradiation, the cells were fed fresh medium until colonies appeared. Colonies were fixed and stained with crystal violet blue, and scored. Five replicate plates were used to determine each survival point, and experiments were repeated at least three times.

Flow cytometric analysis of apoptosis Flow cytometric analysis of apoptosis was carried out at indicated times after addition of cisplatin or UV irradiation, using an ApoAlert Apoptosis detection kit (Clontech, Palo Alto, CA) with FACS Calibur (Becton Dickinson, San Jose, CA) according to the manufacturer's instructions. Fluorescence data were displayed as dot-plots using the Cell Quest software (Becton Dickinson).

Western blotting Cells were washed with PBS and lysed in 20 μl of sodium dodecyl sulfate (SDS) lysis buffer (25 m*M* Tris-HCl pH 6.5, 1% SDS, 0.24 *M* β-mercaptoethanol, 0.1% Bromophenol Blue, 5% glycerol). Following boiling, aliquots (routinely 50%) were subjected to 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to a nitrocellulose membrane, proteins were detected by monoclonal mouse anti-p85 antibody (Upstate Biotechnology, Lake Placid, NY) or β-actin antibody (Sigma Chemical Co., St. Louis, MO) and horseradish peroxide (HRP)-conjugated goat anti-mouse Ig antibody (Amersham Pharmacia, London, UK) using the "Super Signal" CL-HRP Substrate System (Pierce, Rockford, IL). Total Akt (phosphorylated and non-phosphorylated) and phosphorylated Akt were detected using PhosphoPlus Akt Antibody Kit (New England BioLabs Inc., MA) in order to determine the inhibition of PI3-K activity by overexpression of Δp85.

RESULTS

T98G cells overexpressing Δp85 Clones overexpressing Δp85 were identified by a western blot analysis with mouse monoclonal anti-p85 antibody. Two Δp85-overexpressing clones of T98G cells (T98GΔ1 and T98GΔ2) were obtained (Fig. 1A). We evaluated the suppression of the phosphorylation of Akt, a downstream molecule of PI3-K, in order to check the inhibition of PI3-K activity by overexpression of Δp85. Phosphorylation of Akt was detected in cells transfected with the vector alone (T98Gv) following 10 J/m^2 UV irradiation. In contrast, no significant phosphorylation of Akt was shown in T98GΔ1 following UV irradiation (Fig. 1B).

Sensitization of T98G cells overexpressing Δp85 to cisplatin We evaluated the survival of T98GΔ1 and T98GΔ2

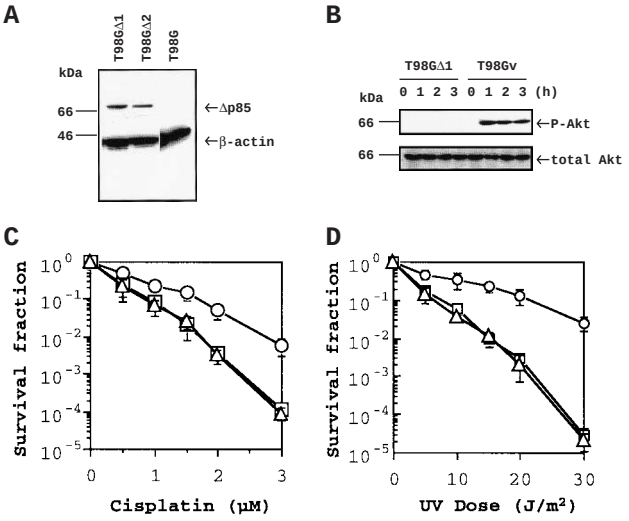

Fig. 1. Sensitivities of T98GΔ1 and T98GΔ2 cells to cisplatin and UV. (A) Western blotting of p85 and β-actin of T98GΔ1, T98GΔ2 and T98G was performed as described in "Materials and Methods." (B) Suppression of PI3-K activity by overexpression of Δp85 was evaluated using PhosphoPlus Akt Antibody Kit. Whole-cell lysates were prepared from T98GΔ1 cells, T98GΔ2 cells (data not shown) and T98Gv cells at the indicated times following UV irradiation or cisplatin treatment (data not shown). Sensitivities of T98GΔ1 and T98GΔ2 cells to cisplatin (C) and UV (D) were assessed with a clonogenic assay as described in "Materials and Methods." All assays were repeated at least three times. Data are expressed as means with corresponding SDs. ○ T98Gv, □ T98GΔ1, △ T98GΔ2.

following cisplatin treatment by means of a clonogenic assay. It was found that T98GΔ1 and T98GΔ2 became markedly more sensitive to cisplatin in a concentration-dependent manner compared to T98Gv cells (Fig. 1C). Overexpression of Δp85 led to a sensitization enhancement ratio (at 10% survival) of 1.9 in T98G cells. The plating efficiencies were comparable between T98Gv and T98GΔ cells (approximately 60%).

Sensitization of T98G cells overexpressing Δp85 to UV Next, we measured the UV sensitivity of T98GΔ1 and T98GΔ2. The transfectants showed much greater sensitivity to UV exposure compared to T98Gv cells (Fig. 1D). The sensitization was dose-dependent. The dose of UV irradiation required for 10% survival was decreased by a factor of approximately 2.8 in T98G cells overexpressing Δp85. The plating efficiencies of T98Gv and T98GΔ cells were comparable (approximately 60%).

Induction of apoptosis of T98GΔ1 cells following cisplatin treatment We determined the mode of cell death in T98G cells overexpressing Δp85 after treatment with 3 *μM* cisplatin by dual parameter flow cytometry with annexin V and propidium iodide (PI) staining (Fig. 2). Though apoptosis was induced after 6 h in both T98Gv and T98GΔ1 cells, T98GΔ1 cells exhibited extensive apoptosis compared to T98Gv cells (Fig. 2C). We followed the percentage of apoptotic cells until 60 h after cisplatin treatment. At all time points, T98GΔ1 cells showed a much greater extent of apoptosis than T98Gv cells.

Induction of apoptosis of T98GΔ1 cells following UV exposure We also checked the apoptotic fraction of T98GΔ1 cells after treatment with 30 J/m^2 UV, by means of flow cytometry (Fig. 3). In both T98GΔ1 and T98Gv cells, apoptosis was induced 6 h following the exposure to UV. However, the apoptotic fraction of T98Gv decreased and returned to the basal level 12 and 48 h after UV exposure, respectively. In contrast, T98GΔ1 underwent a larger extent of apoptosis in response to UV treatment and the apoptotic fraction did not decrease over 60 h (Fig. 3C).

Sensitization of other cell lines overexpressing Δp85 to these genotoxic stresses Finally, in order to address the question of whether other cell lines overexpressing Δp85 are sensitized to these genotoxic stresses or not, we generated human glioblastoma A172 cells and human melanoma G361 cells overexpressing Δp85 (A172Δ1 and G361Δ1, Fig. 4, A and B, respectively). Like T98GΔ1 and

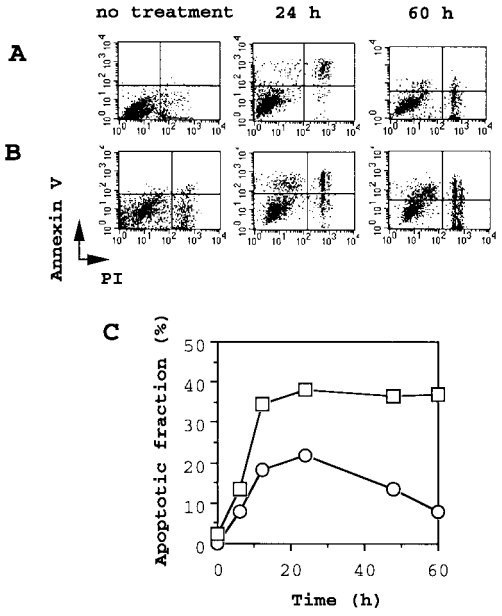

Fig. 2. Flow cytometric analysis of apoptosis induced in T98GΔ1 following 3 *μM* cisplatin treatment. Cells were analyzed using an ApoAlert Apoptosis detection kit and FACS Calibur. Representative data of the induction of apoptosis of T98Gv (A) and T98GΔ1 (B) before and 24 and 60 h after the addition of cisplatin are shown. The relative fluorescence intensity due to PI staining and annexin V-fluorescein isothiocyanate (FITC) is plotted on the log scale *x*- and *y*-axis, respectively. (C) The percentages of apoptotic cells of T98GΔ1 and T98Gv at the indicated times after the addition of cisplatin. ○ T98Gv, □ T98GΔ1.

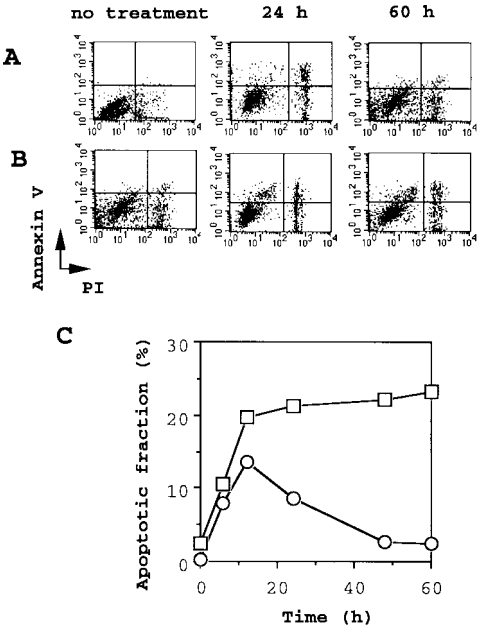

Fig. 3. Flow cytometric analysis of apoptosis of T98GΔ1 following 30 J/m^2 UV. Representative data of the induction of apoptosis of T98Gv (A) and T98GΔ1 (B) before and 24 and 60 h after UV exposure are shown. (C) The percentages of apoptotic cells of T98GΔ1 and T98Gv at the indicated times after the exposure to UV. ○ T98Gv, □ T98GΔ1.

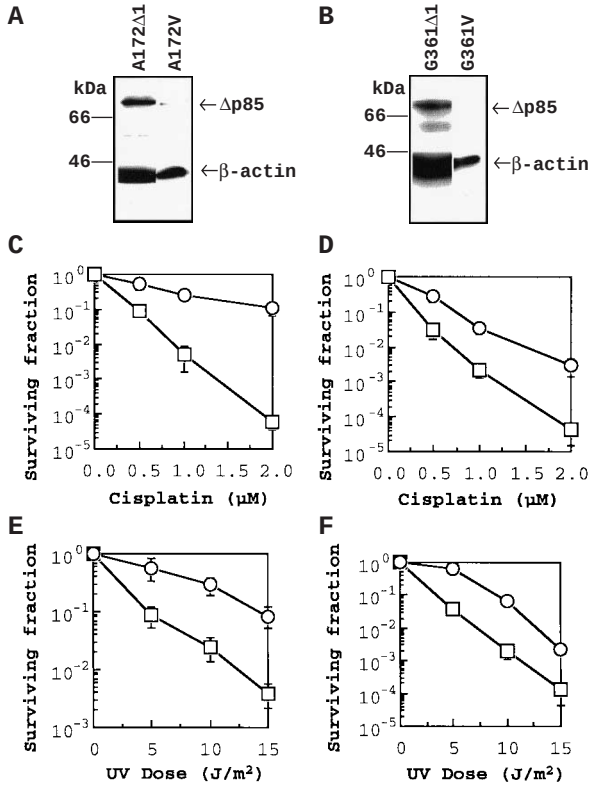

Fig. 4. Sensitivities of A172Δ1 and G361Δ1 to cisplatin and UV. Western blot of p85 and β-actin of A172 (A) and G361 (B) cells overexpressing Δp85 (A172Δ1 and G361Δ1, respectively) and parental cells. Sensitivity to cisplatin and UV (C and E, A172; D and F, G361, respectively) were assessed with a clonogenic assay as described in "Materials and Methods." All assays were repeated at least three times. Data are expressed as means with corresponding standard deviations (SDs). C and E: ○ A172v, □ A172Δ1; D and F: ○ G361v, □ G361Δ1.

T98GΔ2 cells, A172Δ1 and G361Δ1 cells were distinctly sensitized to cisplatin and UV (Fig. 4, C, D and E, F, respectively).

DISCUSSION

Our results showed that T98G cells were sensitized by overexpression of Δp85 to cisplatin treatment or UV exposure. To date, various studies have shown that oncogene and oncosuppressor gene activity can enhance or suppress cellular sensitivity to genotoxic stresses such as chemotherapeutic agents, UV and X-rays in various cell systems.[5–7, 13] The molecules involved in regulation of apoptosis or cell proliferation have been reported to be associated with cellular resistance to cisplatin or UV.[8, 14–16] The catalytic subunit, p110 of PI3-K was also recently shown to be an oncogene important in ovarian cancer by virtue of its frequent amplification in these tumors.[17] The melanoma cells constitutively overexpressing wild-type p85 exhibit an increase in their resistance to UV irradiation.[18] Our results are consistent with these previous reports. This result implies that the disruption of p85/p110 interaction of PI3-K sensitized cells to genotoxic stress and that PI3-K is associated with cellular resistance to these genotoxic stresses.

It has also been demonstrated that a survival signal from the receptor tyrosine kinases utilizes Akt via PI3-K to protect cells from apoptosis.[3, 19] Another report showed that cells expressing kinase-deficient PI3-K undergo more apoptosis following exposure to UV.[19] Overexpression of constitutively activated forms of PI3-K results in a decreased rate of apoptosis in response to serum/growth factor deprivation, UV irradiation or loss of matrix attachment, demonstrating an important role of PI3-K in the regulation of apoptosis.[3, 19–21] We speculated that the suppression of PI3-K leads to the induction of apoptosis, resulting in cellular sensitization to genotoxic stress. Therefore, the induction of cell death in the transfected cells following DNA damage was evaluated by dual parameter flow cytometry with annexin V-FITC and PI (Figs. 2 and 3). Apoptosis was induced to a greater extent in T98GΔ1 than in control T98Gv cells following treatment with cisplatin or UV. Enhanced induction of apoptosis could explain the sensitization of T98GΔ cells, where p85/p110 interaction of PI3-K is disrupted.

We addressed the question of whether PI3-K is involved in the sensitivity of other cells to these genotoxic stresses, and introduced Δp85 into human glioblastoma cells A172 and human melanoma cells G361 (Fig. 4, A and B, respectively). Δp85 also enhanced cell killing of these cells, like T98G cells. The results indicate that sensitization by overexpression of Δp85 is not specific to T98G cells.

In conclusion, we have shown here that the overexpression of dominant-negative Δp85 markedly potentiates the cellular sensitivity of human cancer cells to cisplatin and UV. These results imply that PI3-K is a molecule regulating cellular sensitivity to genotoxic stress and that disruption of the p85/p110 interaction of PI3-K may provide a potential molecular target for cancer treatment.

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