Autophagy Inhibition Can Overcome Radioresistance in Breast Cancer Cells Through Suppression of TAK1 Activation

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Abstract. Background/Aim: Autophagy is frequently activated in radioresistant cancer cells. In the present study, we evaluated the role of autophagy and transforming growth factor-activated kinase 1 (TAK1) in radioresistance. Materials and Methods: TAK1 phosphorylation in MDA-MB231 breast cancer cells was evaluated by western blotting. The regulatory effects of the TAK1 inhibitor and autophagy inhibitor were assessed by cell morphology, cell survival and induction of apoptosis. Results: Radiation induced the phosphorylation of TAK1, whereas the inhibition of TAK1 activity enhanced the cytotoxicity of radiation in MDA-MB231 cells. Autophagy inhibitors significantly enhanced radiation-induced apoptosis of MDA-MB231 cells. This augmentation in radiosensitivity seemed to result from the suppression of TAK1 activation. Conclusion: Inhibition of autophagy enhanced radiosensitivity through suppression of radiation-induced TAK1 activation, suggesting that the modulation of TAK1-induced autophagy may be a good therapeutic strategy to treat radioresistant breast cancer.

Autophagy is a type of cellular catabolic degradation response to nutrient starvation or metabolic stress and has been recognized as a potential mechanism of resistance to chemotherapy and radiotherapy in cancer. Recognition of the protective role of autophagy in tumor cells has generated interest in the possibility that interference with autophagy could overcome resistance and enhance sensitivity to chemoradiotherapy in tumors. Multiple studies in cell culture models, as well as a number of studies in experimental animals, have confirmed that the inhibitors of autophagy facilitate re-sensitization of resistant tumor cells to anticancer treatment (1-14). The use of the autophagy inhibitors 3-methyladenine (3-MA) and chloroquine was shown to confer sensitivity to radiation in ovarian cancer cells through inhibition of autophagy, attenuation of radiation-induced S phase delay, and up-regulation of apoptosis (13). However, the mechanism of autophagy-associated induction of radiosensitivity in cancer cells remains to be established.

Transforming growth factor-beta activated kinase-1 (TAK1), originally identified as a mitogen-activated kinase (MAPK) kinase kinase (MAP3K) activated by transforming growth factor-beta (TGF-β), is a key signaling intermediate regulated by inflammatory factors including interleukin-1 (IL-1) and TGF-beta toll-like receptor (TLR) agonists, and CD40 ligands. Once activated, TAK1 transmits the upstream signal from the receptor by phosphorylating the downstream kinases comprising the MAPKs p38, c-Jun N-terminal kinase (JNK), and I-kappa B kinase (Iκκ) complex. MAPK p38 and JNK control the transcription factor activator protein-1 (AP1), while IκK activates the nuclear factor-kappa B (NF-κB) pathway. TAK1 regulates cell survival, differentiation, and inflammatory responses via a number of specific transcription factors (15). TAK1 activation in cancer cells results in the induction of NF-κB and AP1, followed by inhibition of apoptosis, enhanced resistance to chemotherapeutic drugs, and increased metastasis (16). Alternatively, TAK1 inhibitors have been shown to significantly increase sensitivity to chemotherapy and to promote cancer cell death, suggesting that they can serve as an effective adjunct to current chemotherapeutic regimens for high-risk cancer (16-19). However, the role of TAK1 in the development of radioresistance in cancer remains unknown. Furthermore, although TAK1 promotes autophagic cell death by
suppressing the phosphorylation of p70S6 kinase 1 (20), the correlation of TAK1-induced autophagy with radioresistance is not well-defined.

In the present study we report here that the autophagy inhibitors bafilomycin A1 and chloroquine significantly enhance radiation-induced apoptotic death in MDA-MB 231 breast cancer cells. This augmentation in radiosensitivity seemed to result from suppression of TAK1 activation. To the best of our knowledge, this study provides the first evidence that autophagy inhibition via suppression of TAK1 may enhance the efficiency of radiotherapy.

Materials and Methods

Cell culture. MDA-MB231 breast cancer cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum at 37°C in a humidified atmosphere of 5%.

Irradiation and chemicals. Cells grown to approximately 70% confluence were irradiated using a 6-MV photon beam generated by a linear accelerator (Varian CLINAC 600C) at a dose rate of 2 Gy/min. All reagents were purchased from Sigma-Aldrich (St. Louis, Mo, USA), unless otherwise specified. Cells were treated with the autophagy inhibitors bafilomycin A1 (1 nM) and chloroquine (10 μM), the autophagy inducer rapamycin (50 nM), and the TAK1 inhibitor 5Z-7-oxozeaenol for 1 h, and were then exposed to the indicated dose of radiation.

Cell viability. Following drug or radiation treatment, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, Mo, USA), unless otherwise specified. Cells were treated with the autophagy inhibitors bafilomycin A1 (1 nM) and chloroquine (10 μM), the autophagy inducer rapamycin (50 nM), and the TAK1 inhibitor 5Z-7-oxozeaenol for 1 h, and then exposed to the indicated dose of radiation.

Propidium iodide (PI)/annexin V staining. Apoptosis was quantified using the PI/Annexin V-FTC kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s guidelines. The cell-cycle was analyzed by assaying the uptake of PI (Sigma-Aldrich, St. Louis, Mo, USA) according to the manufacturer’s guidelines. The cell-cycle was analyzed by assaying the uptake of PI (Sigma-Aldrich, St. Louis, Mo, USA) according to the manufacturer’s guidelines.

Western blot analysis. Protein extracts of MDA-MB231 cells treated with 2 Gy (±reagents) and incubated for different times (0, 24, 48, or 72 h) were separated on a 7.5% polyacrylamide gel, followed by transfer to a Hybond™-polyvinylidifluoride (PVDF) membrane (Amersham International Plc., Little Chalfont Buckinghamshire, UK). Membranes were blocked with 5% skimmed milk (Difco Laboratory, Detroit, MI, USA) for 1 h at room temperature, washed with PBS, and incubated overnight at 4°C with primary antibodies against phospho-TAK1 Ser412 (1:1,000; Cell Signaling, Danvers, MA, USA), p62 (1:1,000; MBL), and beta-actin (β-actin; 1:5,000; Sigma-Aldrich, St. Louis, Mo, USA) diluted in 5% bovine serum albumin (BSA) in PBS. Next, membranes were washed with Tween 20-containing PBS (PBST) and incubated with a secondary antibody, anti-mouse horseradish peroxidase (HRP)-conjugated IgG (1:2000; Zymed, San Francisco, CA, USA) in PBST for 1 h. After washing with PBST, specific binding was detected using an ECL kit (Amersham International Plc., Little Chalfont Buckinghamshire, UK) following the manufacturer’s protocol. Following a final wash step, the membrane was developed using a chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA).

Statistical analysis. Statistical analysis was performed using the paired Student’s t-test. Data are presented as mean±SD. Differences with p-values less than 0.05 were considered statistically significant; p-values for each experiment are provided in the Figure legends.

Results

Irradiation-induced autophagy and TAK1 activation represent a survival response that protects MDA-MB231 cells against radiation. We examined the dynamic changes in phospho-TAK1 and p62, a ubiquitin-binding scaffold protein used as a marker of autophagic flux. As shown in Figure 1A, the level of p62 was decreased 24 h after irradiation, indicating an active protein turnover within the autolysosome. However, TAK1 phosphorylation increased from 12 h up to 72 h after irradiation, indicating that TAK1 was activated by radiation treatment. To determine the role of TAK1 activity in radiation-induced cell death, we investigated the effects of 5Z-7-oxozeaenol, an inhibitor of TAK1 kinase activity, on cell survival (Figure 1B). Combined treatment with TAK1 inhibitor and radiation significantly reduced cell survival at 72 h compared to that of cells exposed to radiation or TAK1 inhibitor separately. Collectively, these results suggest that in breast cancer cells, the activation of TAK1-mediated signaling and autophagy may constitute an early protective response against radiation-induced damage, and that TAK1 activation may represent a mechanism that enables cells to acquire radioresistance.

Autophagy inhibition suppresses TAK1 activation in MDA-MB231 cells. Autophagy has been implicated in the development of radioresistance in tumor cells, but its association with TAK1 has not been established. To investigate whether TAK1 activation correlates with autophagy in cancer cells, we examined TAK1 phosphorylation in MDA-MB231 cells treated with an autophagy inducer, rapamycin, and autophagy inhibitors, bafilomycin and chloroquine, by western blotting. As shown in Figure 2, the level of phospho-TAK1 did not change by 12 h after irradiation, indicating that TAK1 was activated by radiation treatment.

Collectively, these results suggest that in breast cancer cells, the activation of TAK1-mediated signaling and autophagy may constitute an early protective response against radiation-induced damage, and that TAK1 activation may represent a mechanism that enables cells to acquire radioresistance.
72 h after treatment with the inhibitors, especially with bafilomycin, which completely blocked the phosphorylation of TAK1. These results suggest that inhibition of autophagy suppresses TAK1 activation in breast cancer cells.

Combination of autophagy inhibitors with radiation increases the radiosensitivity of MDA-MB231 cells. Next, we assessed the effect of autophagy inhibition on the response to irradiation in cancer cells. The regulatory effects of the autophagy inhibitors bafilomycin and chloroquine were assessed by cell morphology (microscopic examination), cell survival (MTT assay), and induction of apoptosis (PI/annexin staining). Analysis of cellular morphology showed that bafilomycin and chloroquine reduced cell proliferation at 48 h (Figure 3A). When combined with irradiation, the autophagy inhibitors, especially chloroquine, significantly reduced cell survival at 48 h compared to that of cells treated with radiation or drugs separately (Figure 3B). When apoptosis was assessed in the PI/annexin V assay, similar results were observed: the combined treatment with radiation and autophagy inhibitors increased apoptotic death in MDA-MB231 cells from 9.82% (radiation alone) to 30.15% (bafilomycin) and 23.94% (chloroquine) (Figure 3C).

Autophagy inhibitors enhance radiosensitivity through suppression of TAK1 activation in MDA-MB 231 cells. To determine whether the enhancement of radiation-induced cancer cell death by the autophagy inhibitors is related to TAK1 activation, we investigated TAK1 phosphorylation in MDA-MB231 cells exposed to combined treatment. As shown in Figure 1, radiation increased the phosphorylation of TAK1, but after the combined treatment with radiation and autophagy inhibitors, TAK1 activation was significantly suppressed (Figure 4). Taken together, these findings suggest
that the inhibition of autophagy enhances radiosensitivity in cancer cells through suppression of TAK1 activation.

Discussion

Radiotherapy is one of the cornerstones in the treatment of patients with breast cancer. Radiotherapy has proven to be very effective for the local control of tumors, and has been shown to greatly reduce the risk of recurrence in women with ductal carcinoma in situ and in patients with node-positive breast cancer (21, 22). However, radiotherapy resistance at relapse strongly contributes to treatment failure. Therefore, strategies to overcome established radioresistance are urgently needed for patients with breast cancer at high risk for relapse. TAK1 is a member of the MAPK family, which is implicated in a variety of signaling pathways that regulate immune and stress responses and inflammation. TAK1-mediated phosphorylation of epidermal growth factor receptor (EGFR) via MAPK p38 in a tyrosine kinase-independent manner suggested a new paradigm for inflammation-related cancer progression (23). In addition, genotoxic agents were shown to induce TAK1-mediated NF-κB activation, indicating a role of TAK1 in resistance to conventional chemotherapy in cancer (16, 17). Indeed, TAK inhibition significantly increased the sensitivity of neuroblastoma and colon and pancreatic cancer cells to chemotherapy-induced cell death (16-18); however, the role of TAK1 in radioresistance has not been investigated. Understanding the molecular mechanisms that mediate radioresistance is critically important for the successful development of curative strategies for patients with high-risk breast cancer. Our results show that the inhibition of TAK1 activity greatly enhanced radiation-induced cytotoxicity in MDA-MB231 breast cancer cells, providing convincing evidence that TAK1 activation is an early protective response to radiation-induced damage. Thus, TAK1-mediated signaling may represent one of the mechanisms that enable cells to develop radioresistance.

Autophagy is a key process that ensures cellular survival during starvation by maintaining cellular energy levels. Activation of autophagy has also been shown to contribute to cancer development by promoting resistance to chemoradiotherapy in tumor cells. Thus, it has been reported that autophagy inhibition enhances radiation-induced apoptosis in many types of cancer cells (1, 3-5, 7, 9, 10, 13, 14, 24, 25). Boya et al. demonstrated that if autophagy was inhibited either genetically or pharmacologically, human breast cancer cell death occurred through apoptosis under conditions of nutrient depletion (26). In our study, direct inhibition of autophagic activity enhanced radiation-induced apoptosis, leading to a significant increase in cell death. Apoptosis is considered an important contributor in the radiation-induced elimination of tumor cells. Our results demonstrated that activation of the TAK1 kinase reduced apoptosis in breast cancer cells, and that

Figure 2. Western blot analysis of p62 expression and transforming growth factor-beta activated kinase-1 (TAK1) phosphorylation in MDA-MB231 cancer cells treated with an autophagy inducer (rapamycin, 50 nM) or autophagy inhibitors (bafilomycin, 1 nM or chloroquine, 10 μM).
the inhibition of autophagy could suppress TAK1 activation, suggesting an association between autophagy and TAK1 signaling in radioresistant cancer cells. In the present investigation, we demonstrated that the autophagy inhibitors bafilomycin and chloroquine promoted radiation-induced cell death in MDA-MB231 cancer cells and could overcome established radioresistance through blocking radiation-induced TAK1 activation.

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