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Glucosamine induces autophagic cell death through the stimulation of ER stress in human glioma cancer cells

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ABSTRACT

Autophagy can promote cell survival or death, but the molecular basis of its dual role in cancer is not well understood. Here, we report that glucosamine induces autophagic cell death through the stimulation of endoplasmic reticulum (ER) stress in U87MG human glioma cancer cells. Treatment with glucosamine reduced cell viability and increased the expression of LC3 II and GFP-LC3 fluorescence puncta, which are indicative of autophagic cell death. The glucosamine-mediated suppression of cell viability was reversed by treatment with an autophagy inhibitor, 3-MA, and interfering RNA against Atg5. Glucosamine-induced ER stress was manifested by the induction of BiP, IRE1α, and phospho-eIF2α expression. Chemical chaperon 4-PBA reduced ER stress and thereby inhibited glucosamine-induced autophagic cell death. Taken together, our data suggest that glucosamine induces autophagic cell death by inducing ER stress in U87MG glioma cancer cells and provide new insight into the potential anticancer properties of glucosamine.

1. Introduction

Macroautophagy, hereafter referred to as "autophagy", is a cellular process in which cytoplasmic material, including organelles, are degraded. During autophagy, cytoplasmic constituents are sequestered into double-membrane vesicles (autophagosomes) and delivered to lysosomes for degradation or recycling [1]. Autophagy is triggered when cells are exposed to stress stimuli such as hypoxia [2], radiation [3], chemicals [4–6], or nutritional deprivation [7]. Several anticancer agents, including arsenic trioxide [4], tamoxifen [8], and rapamycin [9], are known to induce autophagy. The outcome of autophagy activation is dependent on the cellular context, the strength and duration of the stress signals [10–12]. Autophagy produces a form of cell death called autophagic cell death (or Type II programmed cell death) or plays a cytoprotective role depending on the specific circumstance. Although the mechanisms responsible for the dual action of autophagy in cancer cells have not be clarified, autophagy has been proposed to play an important role in both tumorigenesis and cancer cell death [10,11].

Glucosamine, an amino monosaccharide, is widely taken as a dietary supplement to relieve discomfort of osteoarthritis-related joint pain [13]. Glucosamine has been reported to inhibit tumor growth in vitro and in vivo in many studies [14,15]. The mechanisms proposed for the anticancer action of glucosamine involve the inhibition of HIF-1 [16], the disruption of the structure and function of cellular membranes [17], and the inhibition of p70S6K [18]. However, the mechanism for the anticancer effects of glucosamine is still not clearly understood.

It has been previously shown that glucosamine treatment can cause endoplasmic reticulum (ER) stress and promote the activation of the unfolded protein response (UPR) [19,20]. The ER performs multiple cellular functions, including protein folding and trafficking and regulating intracellular calcium concentrations. The disruption of these ER functions causes a cellular condition known as ER stress. The ER stress response triggers the UPR [21,22]. The UPR, a self-protective mechanism, ameliorates protein loading on the ER by both temporarily shutting down protein translation and increasing ER folding capacity through a complex regulation of gene transcription. UPR activation, while having the capacity to protect cells by providing an opportunity to overcome stress, can also induce cell death (apoptotic, autophagic, or both) when the stress is prolonged or severe [22]. Given the links between glucosamine and ER stress and between ER stress and autophagy, we were interested in examining whether glucosamine induces autophagic cell death in cancer cells.

Here, we show that glucosamine induces autophagic cell death through the stimulation of ER stress in U87MG glioma cancer cells. Our findings provide a novel potential mechanism mediating the anticancer activity of glucosamine.

2. Materials and methods

2.1. Cell lines and materials

U87MG human glioma cells and MDA-MB-231 human breast cancer cells were obtained from the American Type Culture

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Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), streptomycin (100 μg/ml), and 100 U/ml penicillin at 37 °C in a humidified 5% CO_2 atmosphere. Glucosamine, 3-methyl adenine (3-MA), 4-phenyl butyric acid (4-PBA), thapsigargin, and anti-β-actin antibody were purchased from Sigma (St. Louis, MO, USA). Benzyloxycarbonylvalyl-alanyl-aspartic acid (O-methyl)-flu oro-methylketone (z-VAD-fmk) was purchased from R&D systems (Minneapolis, MN, USA). Anti-LC3 antibody was obtained from Medical & Biological Laboratories (Nagoya, Japan). Antibodies for Beclin-1 and BiP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-IRE1α antibody and anti-phospho-eIF2α antibody were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Immunoblotting

Whole cell lysates were prepared in a modified RIPA buffer (50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% SDS, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EDTA, 200 nM aprotinin, 20 μM leupeptin, 50 μM phenanthroline, 280 μM benzamidine-HCl). Immunoblotting was performed as previously described [18]. Immunoblotting was detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

2.3. Viable cell counting

For cell count analysis, U87MG cells and MDA-MB-231 cells were seeded at a density of 1×10^5 cells/well and 8×10^4 cells/well, respectively. After 18 h, cells were treated with 0, 0.5, 1, 2, or 5 mM glucosamine for 48 h. After treatment, cells were detached from each well using 0.25% trypsin/EDTA. Trypan blue was then added to the cell suspension. The number of viable cells was counted using a hemocytometer.

2.4. Flow cytometric analysis

For flow cytometric analysis of DNA content, approximately 1×10^6 cells were fixed in 70% ethanol at −20 °C for 24 h. Ethanol-fixed cells were stained with propidium iodide (PI) staining solution (50 μg/ml PI, 0.1 mg/ml RNase A, 0.1% NP-40, 0.1% trisodium citrate) for 30 min and then analyzed by a FACS analyzer (BD Biosciences, Sparks, MD, USA).

2.5. Examination of LC3 translocation

For the analysis of green fluorescent protein-fused LC3 (GFP-LC3) localization, U87MG and MDA-MB-231 cells were transfected with a plasmid encoding GFP-LC3 [23], which was kindly provided by Prof. Tamotsu Yoshimori (Department of Cellular Regulation Research, Institute for Microbial Diseases, Osaka University, Japan). For GFP-LC3 plasmid transfection, U87MG and MDA-MB-231 cells were seeded in growth medium without antibiotics, and plasmid DNA was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 16 h of incubation, the medium was exchanged for complete medium containing 10% serum and antibiotics. The cells were incubated for an additional 48 h, and positive stable clones were selected by growing cells with G418 (1 mg/ml) for 2 weeks. Stable GFP-LC3 transfectants cells were seeded in 2-well chamber slides at a density of 4×10^4 cells/well, and after 18 h, cells were then treated with glucosamine for 48 h. Cells were then washed with PBS three times and fixed with 4% paraformaldehyde solution in PBS for 5 min at room temperature. Cells were again washed with PBS three times and mounted with fluorescent mounting medium (Dako, Glostrup, Denmark). The images were obtained using a fluorescence microscope (Leica DM 3000, Wetzlar, Germany).

2.6. RNA interference of Atg5

Small interfering RNAs (siRNAs) against Atg5 and scrambled siRNA were obtained from Santa Cruz Biotechnology. Cells cultured in 6-well plates were transfected with siRNA at a 150 nM final concentration using Lipofectamine 2000 (Invitrogen) according to the supplier's protocol. After 5 h, the medium was changed to complete medium, and cells were incubated for another 48 h before treatment with glucosamine. Cells were then harvested for counting and subjected to western blot analysis.

2.7. Statistical analysis

Data represent means ± SD of three independent experiments. Statistical analysis was performed by Student's *t*-test at a significance level of $P < 0.05$.

3. Results

3.1. Glucosamine induces autophagic cell death

To gain insight into the mechanism by which glucosamine exerts cytotoxic effects in cancer cells, U87MG and MDA-MB-231 cells were treated with glucosamine, and cell viability and cell cycle analyzes were performed. Treatment with glucosamine resulted in reduced cell viability in a dose-dependent manner in both cell lines (Fig. 1A). In flow cytometric analysis, glucosamine did not lead to the accumulation of a sub-G1 fraction, suggesting that the cytotoxicity of glucosamine was not associated with the induction of apoptosis (Fig. 1B). To ensure that the cytotoxic effect of glucosamine was independent of apoptosis, cells were treated with the pan-caspase inhibitor z-VAD-fmk in addition to glucosamine (Fig. 1A). Treatment with z-VAD-fmk did not have any significant effect on the glucosamine-mediated reduction of cell viability, suggesting that caspase-dependent cell death is not involved in glucosamine-mediated cell death.

To determine the mechanism by which glucosamine exerts cytotoxic effects, we tested whether glucosamine can induce autophagy, which is known as a non-apoptotic cell death mechanism. LC3 is essential for autophagosome formation [23] and exists in two forms. During autophagy, LC3 I residing in the cytosol is specifically processed to the membrane-bound LC3 II. The accumulation of LC3 II is considered one of the hallmarks of autophagy [1]. Treatment with glucosamine increased the expression level of LC3 II in a time- and dose-dependent manner (Fig. 2A), suggesting that glucosamine can induce autophagy. The intracellular localization of LC3 in autophagic vacuoles can be studied by transfecting cells with an expression plasmid of GFP-LC3 [23,24]. Fluorescence microscopy experiments were performed to examine LC3 translocation during autophagosome/autolysosome formation. In U87MG and MDA-MB-231 cells stably expressing GFP-LC3, GFP-LC3 was observed as diffuse green fluorescence in the cytoplasm (Supplementary Fig. 1). In contrast, punctate fluorescence patterns were observed in glucosamine-treated cells. The number of cells with punctate green fluorescence increased significantly after treatment with glucosamine in both U87MG and MDA-MB-231 cells, indicating that glucosamine could induce autophagy (Fig. 2B).

Because autophagy has been implicated in both the promotion and inhibition of cell death, we examined its participation in the cytotoxic effects of glucosamine. To this end, we examined the effects of 3-MA as a pharmacological inhibitor of autophagy and small interfering RNA against Atg5, which is an essential protein in the formation of the autophagosome [1] in glucosamine-induced

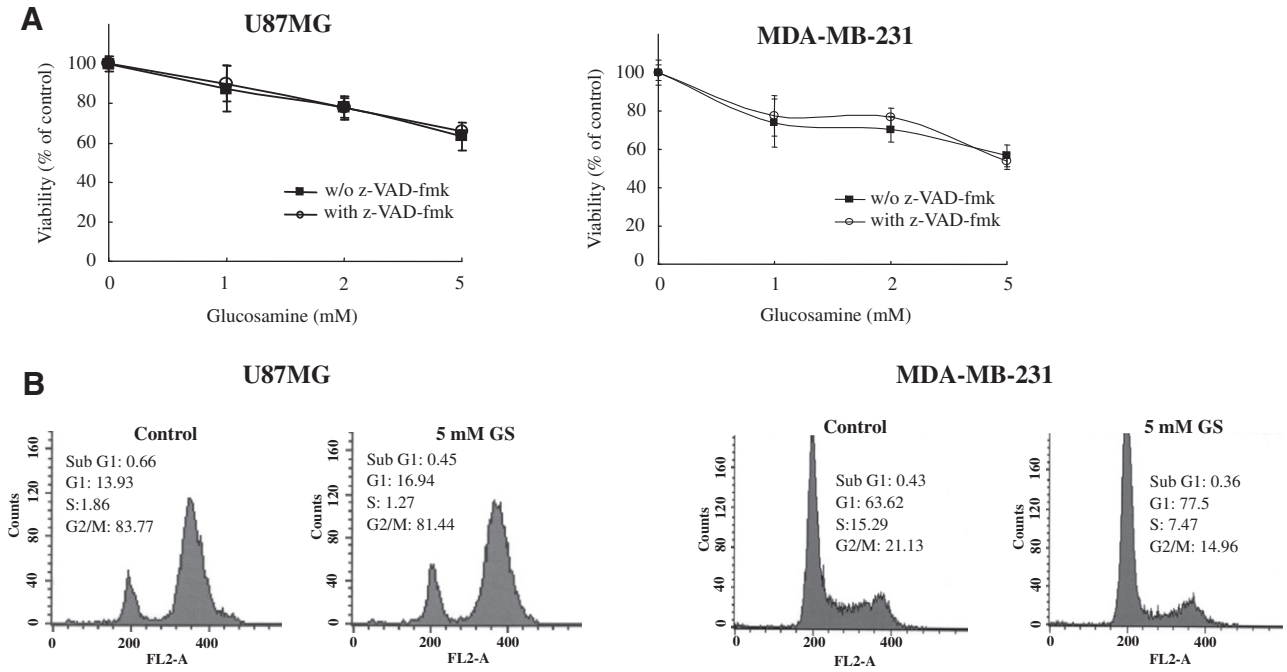

Fig. 1. Glucosamine reduces cell viability in cancer cells through a caspase-independent mechanism. (A) U87MG and MDA-MB-231 cells were treated with glucosamine in the presence or absence of z-VAD-fmk for 48 h. Cell viability was determined by cell counting as described in the Section 2. (B) Cells were treated with 5 mM glucosamine for 48 h, and their cell cycle distribution was determined by flow cytometric analysis of the DNA content after staining with propidium iodide.

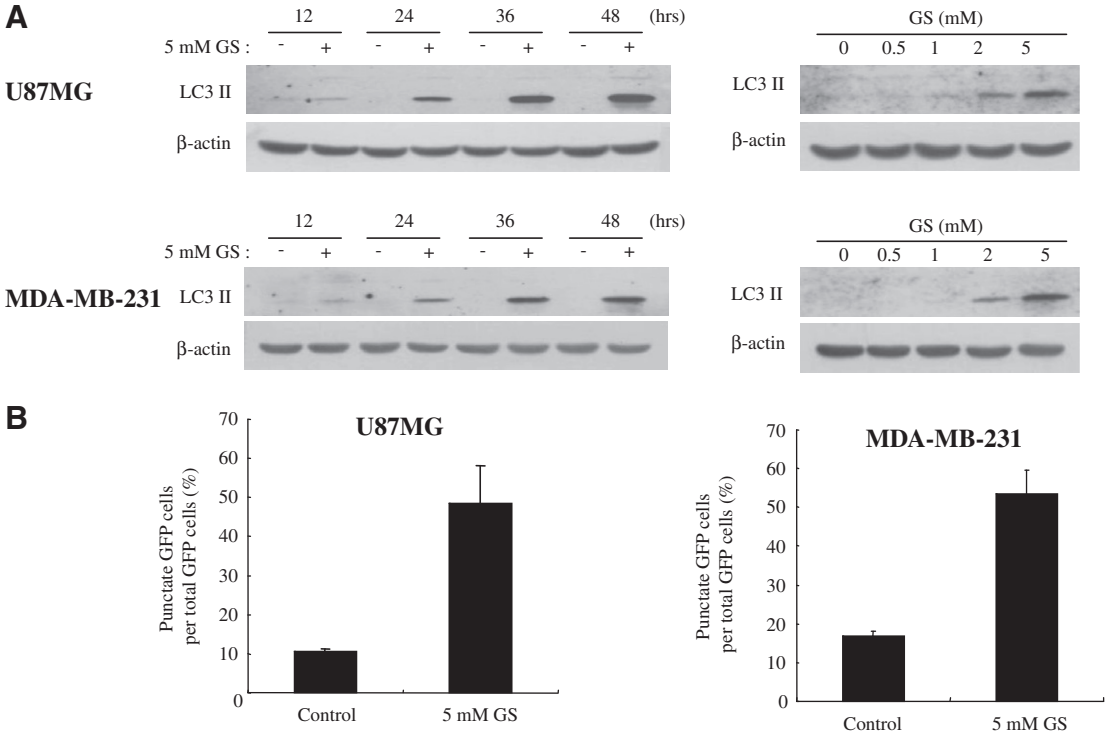

Fig. 2. Glucosamine induces autophagy. (A) Glucosamine induced the expression of LC3 II. U87MG and MDA-MB-231 cells were treated with 5 mM glucosamine for the indicated times (left) or treated with various concentrations of glucosamine for 48 h (right). After treatment with glucosamine, the whole cell lysates were analyzed by immunoblotting. (B) U87MG and MDA-MB-231 cells stably expressing GFP-LC3 were incubated in the absence or presence of 5 mM glucosamine for 48 h and analyzed by fluorescence microscopy. The percentage of cells with punctate GFP-LC3 fluorescence was calculated relative to all GFP-positive cells by direct counting of approximately 100 cells in three different fields.

cell death. Both 3-MA and Atg5 siRNA strongly reduced glucosamine-induced autophagy and cell death in U87MG cells (Fig. 3). Treatment with 3-MA (10 mM) in U87MG cells challenged with glucosamine led to a decrease in the glucosamine-induced expression of LC3 II (Fig. 3A), thereby strongly inhibiting cell death (Fig. 3B). The selective knockdown of Atg5 expression by RNA

interference resulted in the inhibition of glucosamine-induced LC3 II expression and reduced the number of punctate GFP-LC3 cells (Fig. 3C and D). Coinciding with the reduction of autophagy, glucosamine-induced cell death was strongly reduced (Fig. 3E). The survival advantage achieved with both 3-MA and Atg5 siRNA in the glucosamine-induced cell death of U87MG cells provided evidence that glucosamine induces not only autophagy but also autophagic cell death, which contributes to the role of glucosamine in inducing cell death. Taken together, these data suggest that autophagy plays a significant role in glucosamine-induced cancer cell death.

3.2. Glucosamine induces autophagic cell death through ER stress

It has been reported that glucosamine can cause ER stress [19,20]. Because ER stress has been implicated in promoting autophagy, we investigated whether glucosamine-induced autophagy is linked to ER stress. To this end, we tested glucosamine activity in the induction of ER stress in U87MG cells. Treatment with glucosamine induced the expression of ER stress response markers, including BiP, IRE1α, and phospho-eIF2α, in a dose-dependent manner (Fig. 4A). Thapsigargin (TG) was used as a positive control of ER stress. These results suggest that glucosamine induces ER stress. We next investigated whether the activation of ER stress is involved in the induction of autophagy in response to glucosamine treatment in U87MG cells. To this end, we examined the effects of 4-PBA in ER stress response and autophagy induced by glucosamine, as 4-PBA is a chemical chaperone that can reduce the ER stress response through stabilizing protein conformation [25,26]. Treatment with 4-PBA in U87MG cells challenged with glucosamine resulted in reduced glucosamine-induced expression of BiP (Fig. 4B). Coinciding with the reduction of BiP expression, 4-PBA diminished LC3 II expression (Fig. 4B) and punctate GFP-LC3 formation (Fig. 4C). These findings suggest that glucosamine induces autophagy through the activation of the ER stress response. Moreover, treatment with 4-PBA reduced cell death induced by glucosamine (Fig. 4D). Taken together, these data suggest that glucosamine induces autophagic cell death by stimulating ER stress in U87MG cells.

4. Discussion

The primary role of autophagy in cancer is still unclear. Depending on the cellular context, the strength and duration of the triggering stimuli, autophagy can promote cell survival or cell death [10,12,27]. The data presented here demonstrated that the induction of autophagy by glucosamine leads to cancer cell death. Glucosamine treatment did not induce apoptosis but rather induced autophagy manifested by LC3 II expression and GFP-LC3 puncta formation in U87MG cells. Coincident with glucosamine-induced autophagy, the cell viability of U87MG cells was decreased. Moreover, experiments performed with 3-MA and Atg5 siRNA restored the cell viability that was compromised by glucosamine, but co-treatment of z-VAD-fmk did not enhance cell viability. These results strongly suggest that autophagy induced by glucosamine promotes autophagic cell death rather than apoptotic cell death. The resistance of cancer cells to apoptosis constitutes an important clinical problem in cancer therapy. As an alternative cell death pathway to apoptosis, autophagy is currently considered to be an important research target for therapy [28]. Our findings suggest that glucosamine is a cytotoxic agent that could be added to the list of autophagy-inducing agents with potential as cancer chemotherapeutic agents.

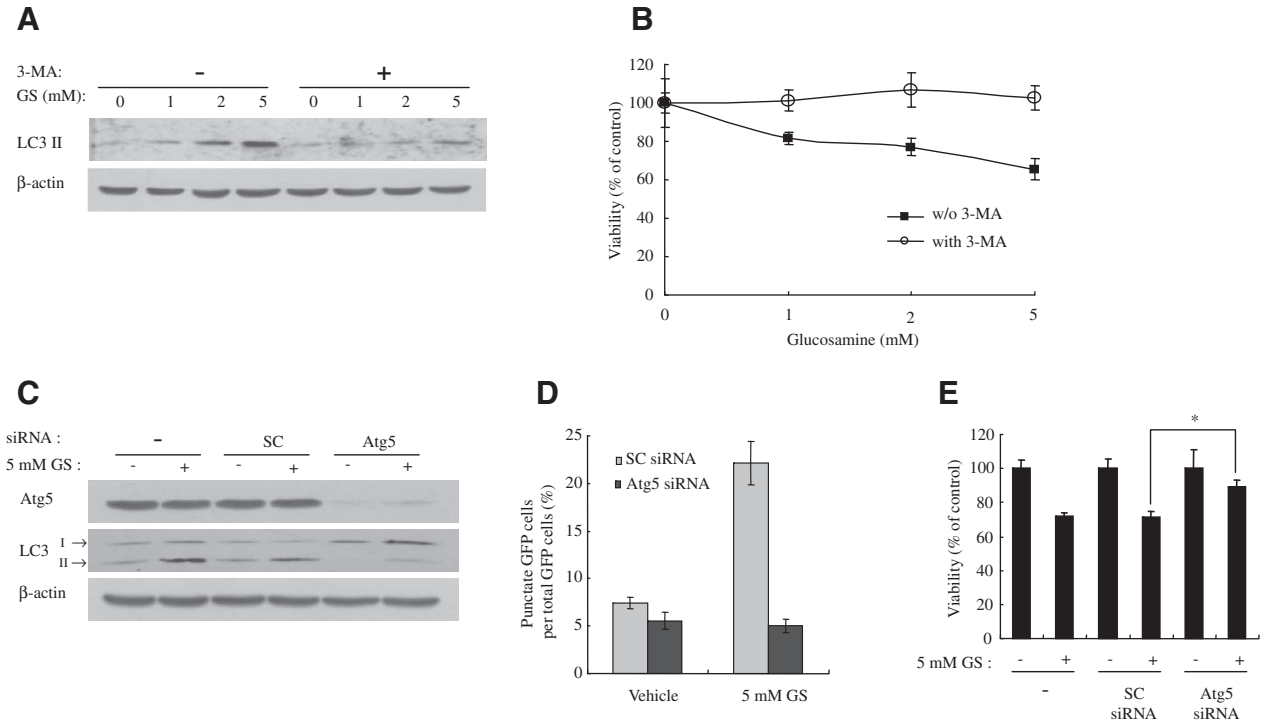

Fig. 3. Glucosamine induces autophagic cell death. U87MG cells were treated with various concentrations of glucosamine in the absence or presence of 3-MA. (A) After 48 h, the whole cell lysates were subjected to immunoblotting, and (B) cell viability was determined by cell counting as described in the Section 2. U87MG cells transfected with scrambled control siRNA or Atg5 siRNA were treated with 5 mM glucosamine for 48 h. (C) The expression of Atg5 and LC3 II protein were determined by immunoblotting. U87MG cells stably expressing GFP-LC3 were transfected with scrambled control siRNA or Atg5-specific siRNA and then treated with 5 mM glucosamine for 48 h. The cells were analyzed by fluorescence microscopy. (D) The percentage of cells with punctate GFP-LC3 fluorescence was calculated relative to all GFP-positive cells by direct counting of approximately 100 cells in three different fields. U87MG cells transfected with scrambled control siRNA or Atg5-specific siRNA were treated with 5 mM glucosamine for 48 h, and (E) cell viability was determined by cell counting. *, indicates a significant difference of cell viability ($p < 0.05$).

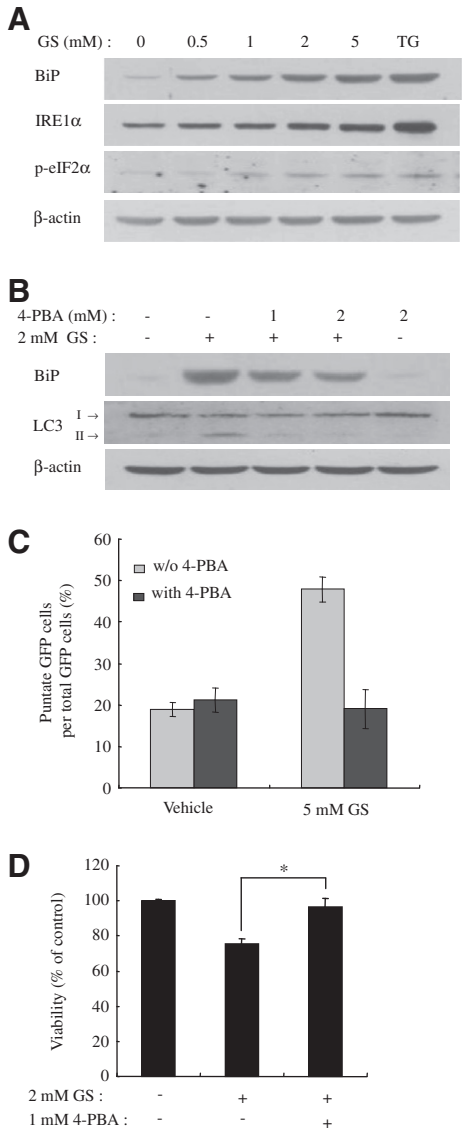

Fig. 4. Glucosamine induces autophagic cell death by stimulating ER stress. (A) U87MG cells were treated with the indicated concentrations of glucosamine for 48 h. The whole cell lysates were analyzed by immunoblotting. (B) U87MG cells were treated with 2 mM glucosamine in the absence or presence of the chemical chaperone 4-PBA for 48 h. The whole cell lysates were analyzed by immunoblotting. (C) U87MG cells stably expressing GFP-LC3 were treated with 5 mM glucosamine in the absence or presence of 2 mM 4-PBA for 48 h and analyzed by fluorescence microscopy. The percentage of cells with punctate GFP-LC3 fluorescence was calculated relative to all GFP-positive cells by direct counting of approximately 100 cells in three different fields. (D) U87MG cells were treated with 2 mM glucosamine for 48 h in the absence or presence of 4-PBA, and cell viability was determined by cell counting. *, indicates a significant difference of cell viability ($p < 0.05$).

Previously, several studies have shown glucosamine-induced ER stress in various cell types [19,20]. In accordance with these reports, glucosamine induced ER stress, as shown by the induction of BiP, IRE1α, and phospho-eIF2α expression in U87MG cells. The mechanism of glucosamine-induced ER stress is unknown. In this study, we showed that ER stress signaling participates in the mechanism of glucosamine-induced autophagic cell death. Recently, accumulating evidence has suggested that ER stress is linked to autophagy [29,30]. The outcome of ER stress-mediated autophagy varies depending on the cell type and stimulus. Several reports have shown that ER stress-induced autophagy may play an important role in cell survival [29,31,32]. In contrast, excessive or prolonged stress results in autophagic cell death [33–35]. In this study, glucosamine-induced ER stress triggered autophagic cell death rather than playing a cytoprotective role. The molecular mechanisms responsible for this action have not been investigated in this report, and further evaluation is needed.

In summary, this study found that glucosamine induces autophagic cell death by stimulating ER stress. These findings provide new insight into a potential mechanism of the anticancer properties of glucosamine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.07.050.

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