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1-Deoxymannojirimycin, the α1,2-mannosidase inhibitor, induced cellular endoplasmic reticulum stress in human hepatocarcinoma cell 7721 ☆

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Abstract

α1,2-Mannosidases, key enzymes in *N*-glycan processing and located both in the endoplasmic reticulum and golgi, have been targets in the development of anti-cancer therapies. Previous studies have shown its involvement in protein degradation. In this study, 1-deoxymannojirimycin, a specific inhibitor of α1,2-mannosidase and generating 'high mannose' type of *N*-glycan, was treated in human hepatocarcinoma 7721 cells and induced the endoplasmic reticulum stress. Key moleculars as XBP1 and GRP78/Bip were activated and up-regulated, which suggested the UPR pathway was activated. The cleavage of caspase-12, -9, and -3 was also detected, which implicated the ER stress was triggered and apoptosis occurred in H7721 cells. The results indicate the 'high Man' structure generated by 1-deoxymannojirimycin may constitute potential novel mechanism for ER stress and caspase-12 pathway of cell apoptosis.

Keywords: α1,2-Mannosidase; 1-Deoxymannojirimycin; Endoplasmic reticulum stress; Apoptosis

The N-linked oligosaccharide moieties present on many endoplasmic reticulum synthesized proteins have been shown to play a crucial role in the quality control which guarantees the endoplasmic reticulum accumulation of misfolded proteins in the lumen [1]. Branched chain N-linked oligosaccharides are cotranslationally added to luminal asparagine residues of proteins as preassembled $Man_9GlcNAc_2$ precursors. ER and Golgi α1,2-mannosidases, which are classified as class I α-mannosidases specifically hydrolyze α1,2-mannose residues, catalyze the trimming of the 'high mannose' chains involving four α1,2-linked mannose residues, and this progress generates $Man_5GlcNAc_2$. The subsequent action of GlcNAc transferase I initiates complex chain formation and yields the substrate for Golgi α-mannosidase II which trims the terminal α1,3- and α1,6-mannose residues. By using specific reagents to inhibit these enzymes, the process can be blocked at the different stages. 1-Deoxymannojirimycin (DMJ), a mannose analogue, specifically inhibits the class I α-mannosidases, resulting in the accumulation of glycoproteins containing mainly high mannose type *N*-glycan ($Man_8GlcNAc_2$), while swainsonine (SW) specifically inhibits the class II α-mannosidases in the golgi [2–4]. DMJ, specifically increasing the high mannose type on cells, was treated in H7721 cells, a human hepatocarcinoma cell line, on the cell surface of which complex type *N*-glycans are up to 90% of all total *N*-glycans [5].

Endoplasmic reticulum stress is induced when functions of the endoplasmic reticulum are affected by various intracellular and extracellular stimuli [6]. Under ER stress, unfolded or misfolded proteins accumulate in the lumen of the ER, leading to the unfolded protein response (UPR). Activated genes possessing a UPR element which

☆ *Abbreviations:* DMJ, 1-deoxymannojirimycin; GRP78/Bip, glucose regulated protein 78; XBP1, X box binding protein 1; CHOP, C/EBP homologous protein; PBS, phosphate-buffered saline; HRP, horseradish peroxidase.

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controls the levels of molecular chaperones, such as GRP78/Bip, involved in protein folding in the ER are up-regulated in order to cope with ER stress. When the UPR is unable to rescue the cells, various apoptotic pathways are activated. The UPR activates the transcription of CHOP, which is closely associated with cell death [7,8]. Caspase-12, specifically located on the outer moiety of the ER membrane, is considered as a key caspase family member involved in ER stress mediating apoptosis [9].

Previous evidence of a role for mannosidase activity was limited to study yeast or some trans-membrane protein in degradation [10,11]. It is thought that glycans lacking the terminal mannoses are poorer substrates of glucosidase II and UDP-glucose: glycoprotein glucosyltransferase, thereby bringing the calnexin cycle to an end. Here we focused on the effect of blocking α-mannosidases I in human hepatocarcinoma 7721 cells concerning with ER stress. Some specific stimulant agents are well known to be used in inducing ER stress, such as tunicamycin (glycosylation inhibition), DTT (reductive stress), and thapsigargin (ER Ca^{2+} depletion) [6,12,13]. We observed that the specific inhibitor DMJ can also be a radical stimulus in inducing ER stress.

Experimental procedures and materials

Chemicals. DMJ was purchased from Sigma. Polyclonal anti-CHOP antibody (rabbit anti-human), anti-GRP78 antibody (goat anti-human), and anti-XBP1 antibody (rabbit anti-human) were purchased from Santa Cruz Technology. Polyclonal anti-caspase-3 antibody (rabbit anti-human), anti-caspase-9 antibody (rabbit anti-human), and anti-caspase-12 antibody (rabbit anti-human) were purchased from Cell Signaling Technology.

Cell culture. H7721 cells were obtained from the Institute of Cell and Biochemistry Research of Chinese Academy of Science. Cells were cultured in RPMI-1640 medium supplemented with 10% newborn bovine serum at 37 °C, in a humidified atmosphere of 5% CO_2, treated with penicillin (100 UI/ml) and streptomycin (100 μg/ml).

DMJ treated. The concentration of DMJ (dissolved in PBS) in the culture medium was 1 mM, and the cells were harvested after 8 and 12 h, respectively [14]. H7721 cells without DMJ treatment were used as negative.

RT-PCR. Total RNA (5 μg) extracted from H7721 cells treated with and without DMJ, respectively, after 8 h by Trizol (Gibco) reagents according to the manufacturer's instructions was used as a template for cDNA synthesis. Reverse transcription was carried out by M-MuLV (promega) and then the cDNA was subjected to PCR. Primers used for PCR were as follows: GRP78/Bip forward primer 5′-CTGGGTACAT TTGATCTGACTGG-3′ and reverse primer 5′-GCATCCTGGTGGCTTTCCAGCCATTC-3′ [15]; XBP-1 forward primer 5′-CCTTGTGTAGTTGAGAACCAGG-3′ and reverse primer 5′-GGGGCTTGGTATATATGTGG-3′ [16]; β-actin forward primer 5′-TGGGCATGGGTCAGAAGGAT-3′ and reverse primer 5′-AAGCATTTGCGGTGGACGAT-3′. The PCR products were electrophoresed on agarose gel, visualized by ethidium bromide (EB) staining on image system.

Western blotting analysis. Proteins for GRP/78, XBP1, and CHOP detection were extracted at 8 h, those for caspase-12, caspase-3, and caspase-9 detection were at 12 h [8,17]. Cells treated with and without DMJ were rinsed twice in ice-cold PBS followed by lysis in 1× SDS buffer with 1% PMSF. Proteins bands were quantified with the Improved Lowry method and then applied to SDS–PAGE. After electrophoresis, proteins were blotted to PVDF membranes and then blocked with 5% skim milk powder with 0.1% Tween 20. Subsequently, membranes were incubated with polyclonal GRP78 antibody (1:1000), XBP1 antibody (1:500), caspase-9 (1:1000), caspase-12 (1:1000), caspase-3 (1:1000), and CHOP (1:500), respectively. Incubated at 4 °C overnight, rinsed by TBST (0.1% Tween 20, TBS) three times, the PVDF membranes were incubated with HRP-conjugated bovine anti-goat IgG or bovine anti-rabbit IgG (1:1000), respectively, at room temperature for 2 h. Positive bands were detected using ECL reagents.

Results

Expression of key molecules of ER stress-UPR, GRP78/Bip, XBP1, and CHOP, is induced by DMJ

To study if DMJ can cause ER stress, we first analyzed the expression levels of GRP78/Bip. Induction of the GRP78/Bip promoter is a general response to ER stress, part of the UPR pathway, and is mostly mediated by the transcription factor ATF6. ATF6 is activated via proteolysis, which permits a rapid response to ER stress. The RT-PCR and Western blotting analyses indicated that GRP78/Bip in H7721 treated with DMJ was markedly up-regulated at both transcriptional and translational levels, which were about 1.8 and 1.5 times higher than those in H7721 negative (Figs. 1A–D).

The activation of transcription factor XBP1 is the critical step of the IRE1 pathway induced by ER stress. Activated IRE1 mediates frame-switching splicing of the XBP1, which results in the formation of a potent trans-activator that up-regulates its own expression and that of molecular chaperons. To evaluate the possible role in DMJ induced ER stress, the XBP1 was examined by RT-PCR with primers that permit the detection of both spliced and unspliced (intron-containing) mRNAs. The expected fragments amplified encompassed the overlapping region of two open-reading frames existing in XBP1 mRNA before and after splicing. These two bands with 26 bp difference as expected were 442 and 416 bp, respectively, representing the spliced and unspliced mRNAs of XBP1 ($XBP1^s$ and $XBP1^u$). They were observed both in negative control and H7721 treating with DMJ (Fig. 2A). The proteins, translated from the $XBP1^s$ and $XBP1^u$, were visualized as the bands of 54 kDa $pXBP1^s$ and 33 kDa $pXBP1^u$, respectively (Fig. 2C).

To further verify that DMJ contributes much to ER stress in H7721 cells, we analyzed the translational level of CHOP, which has been implicated in mediating apoptosis during ER stress and is known to be regulated by PERK/eIF-2α. As the Fig. 3 indicate, the translational level of CHOP in H7721 treated with DMJ was obviously higher than that in the H7721 negative, which was about 1.5 times (Fig. 3B). CHOP protein was increased markedly at 8 h and remained little changed up to 18 h (data not shown).

Apoptosis analysis of the H7721 treating with DMJ

As the DMJ caused severe ER stress in H7721, we examined the activation of caspases in H7721. Focusing on

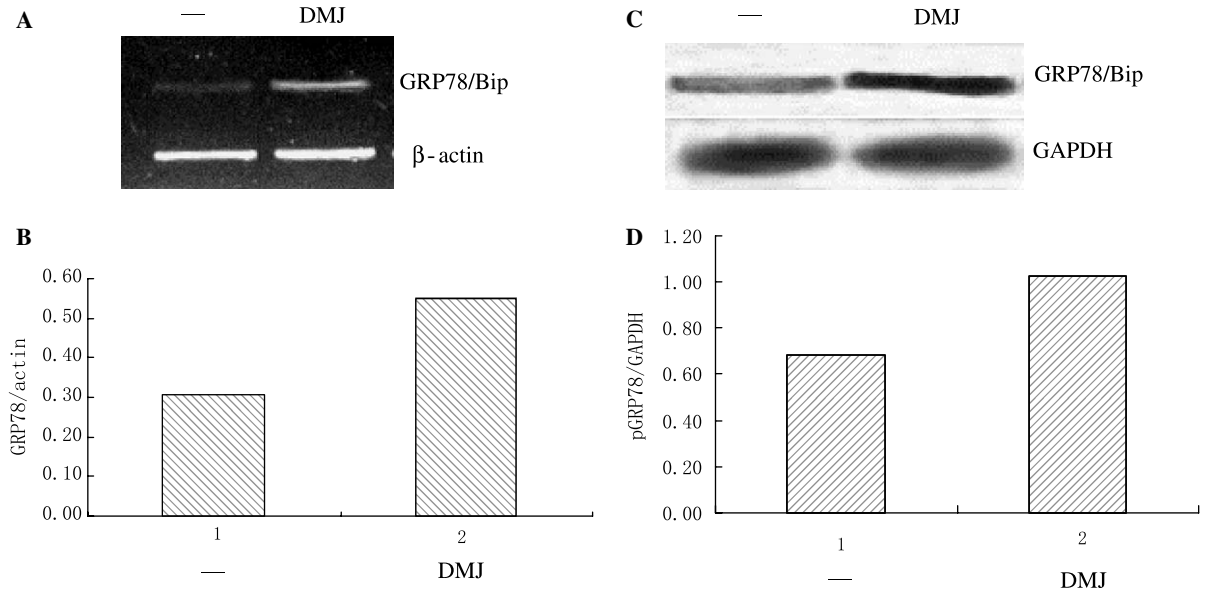

Fig. 1. Expression of GRP78/Bip in H7721 cells treated with DMJ; H7721 cells were maintained under basal conditions or were treated with DMJ (1 mM) for 8 h. Then total mRNAs from these cells were analyzed by RT-PCR for GRP78/Bip and β-actin mRNA, total proteins were also extracted from these cells and analyzed by Western blotting for GRP78/Bip and GAPDH. (A) DMJ induced the expression of GRP78 mRNA in H7721 cells, (B) quantification of relative GRP78 mRNA expression in different cells: 1:H7721, 2:H7721 treated with DMJ for 8 h, (C) DMJ induced the expression of GRP78/Bip protein in H7721 cells, and (D) quantification of relative pGRP78/Bip expression in different cells: 1:H7721, 2:H7721 treated with DMJ for 8 h.

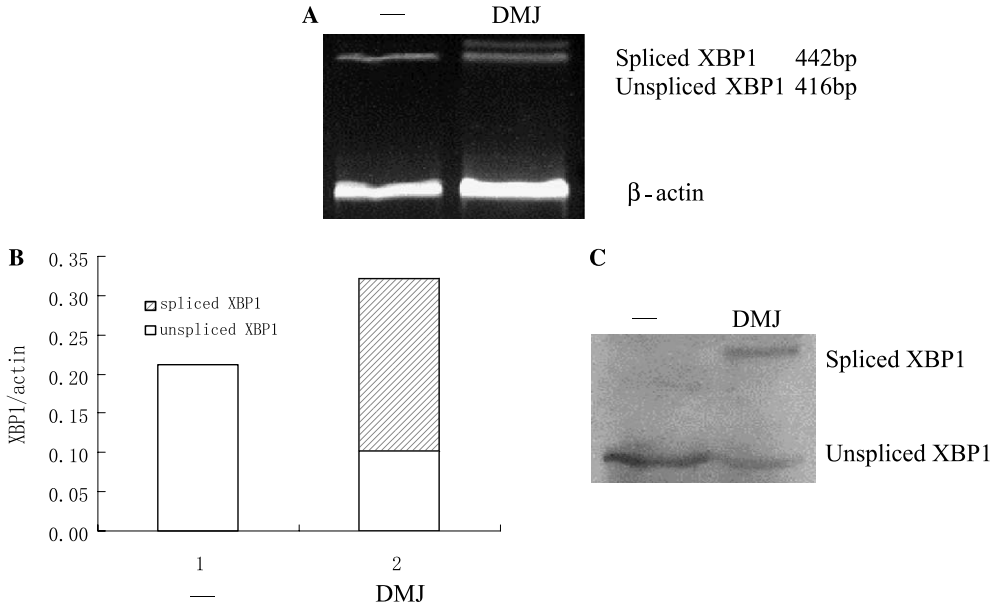

Fig. 2. Splicing of XBP1 in H7721 cells treated with DMJ; H7721 cells were maintained under basal conditions or were treated with DMJ (1 mM) for 8 h. Then total mRNAs from these cells were analyzed by RT-PCR for XBP1 and β-actin mRNA, total proteins were also extracted from these cells and analyzed by Western blotting for XBP1 and GAPDH. (A) DMJ induced the splicing of XBP1 mRNA in H7721 cells, (B) quantification of relative XBP1 mRNA expression in different cells: 1:H7721, 2:H7721 treated with 1 mM DMJ for 8 h, and (C) DMJ induced the splicing of XBP1 protein in H7721 cells.

upstream caspases, we detected that caspase-12, residing normally within the ER, became cleaved during the early course after the DMJ treatment in H7721, which occurred at 12 h. As seen in Fig. 4, activation of caspase-12 was shown by the appearance of the cleaved fragment, and this was accordingly accompanied by a reduction in procaspase-12 levels (Fig. 4A). These data showing low protein levels indicate the processing of caspase-12 appeared after DMJ treatment in H7721 cells. In addition to caspase-12 activation, ER stress stimulated caspase-9 and caspase-3 activation in H7721 as well. As shown in Fig. 3, the proforms of caspase-9 were slightly decreased and the pro-

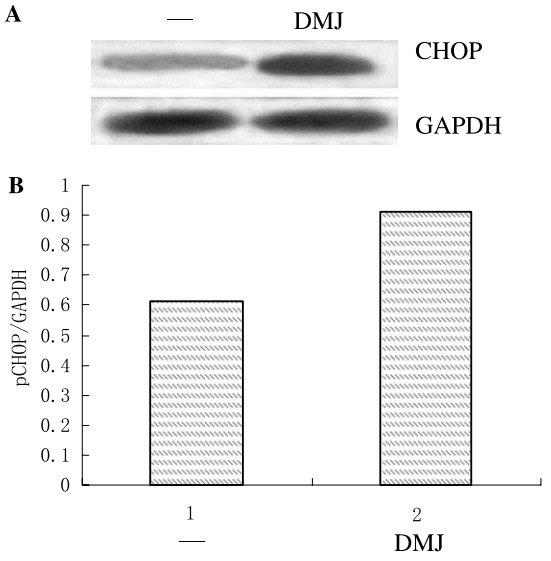

Fig. 3. Western blotting for CHOP; H7721 cells were maintained under basal conditions or were treated with DMJ (1 mM) for 8 h and then total proteins from these cells were analyzed by Western blot for CHOP and GAPDH. (A) DMJ induced the expression of CHOP protein in H7721 cells, (B) quantification of relative pCHOP in different cells: 1:H7721, 2:H7721 treated with DMJ for 8 h.

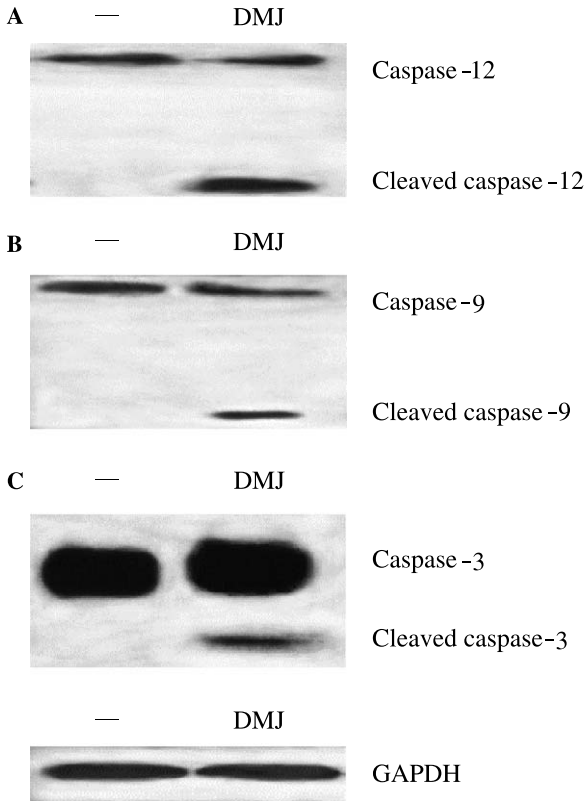

Fig. 4. Activation of caspase-12, -9, and -3 in H7721 cells; H7721 cells were maintained under basal conditions or were treated with DMJ (1 mM) for 12 h and then total proteins from these cells were analyzed by Western-blotting. (A) Activation of caspase-12 in H7721 cells, (B) activation of caspase-9 in H7721 cells, (C) activation of caspase-3 in H7721 cells.

cessed fragment appeared (Fig. 4B). Similarly, as an effective caspase in the cell apoptosis downstream pathway, caspase-3 was also activated and the processed fragment was detected (Fig. 4C).

Discussion

In eukaryotic cells, N-linked glycosylation pathway is responsible for proper processing of proteins as they are synthesized in the ER and golgi apparatus, and concerns ER quality control. This has been analyzed predominantly with regard to removal of glucose residues in calnexin and calreticulin mediated folding in the ER [18]. Previous evidence of a role for mannosidase activity in determining the fate of proteins in the ER was limited to a study on mutant α-antitrypsin or targeting some normal trans-membrane protein such as, CD3-δ, for proteasomal degradation, which suggested to keep the protein from degradation [19,11]. Our results established a role that hasn't been reported before for α1,2-mannosidases activity which concerns with ER stress.

In this study, UPR pathway was activated and triggered by the activation of some sensor proteins. Key chaperones such as XBP1, GRP78/Bip are activated and up-regulated. Mannose residues remained untrimmed and ER stress occurred. In addition to ER stress, H7721 cells treated with DMJ presents the apoptosis phenomenon. We detected caspase-12, as the upstream caspase member considered to be concerned with apoptosis mediated by ER stress, was cleaved, and activation of some downstream caspase members such as caspase-9 and caspase-3. The activation of caspases suggested that ER stress was triggered after treating with α1,2-mannosidase inhibitors. In comparison to our previous study, ER stress was also induced in AsGnTV-H7721 cells in which GnT-V located in golgi was blocked, but it was chronic and there were no signals of apoptosis [20]. Thus, DMJ can also be a radical stimulus that induces ER stress and apoptosis mediated by the stress.

DMJ specifically inhibits the α1,2-mannosidases in both ER and Golgi, generating 'high mannose' type of N-linked glycoprotein in H7721 cells. It is reported that an up-regulation of more complex type of *N*-glycans is present as the most common changes in glycoproteins of tumor cells [21]. While in H7721 cells treated with DMJ, the untrimmed mannose residues disabled the *N*-glycans developing to larger, more branched N-linked oligosaccharides. The high mannose N-glycosylation status may interfere with the chaperons and *N*-glycan synthesis enzymes, which activity may be inhibited and be disfunction in H7721 cells. There is evidence that trimming of mannose residues by α-mannosidases I is important to target misfolded glycoproteins for degradation, though the mechanism remains unclear [22]. In both human and yeast cells, trimming of mannose residues from N-linked oligosaccharides determines the fate of misfolded glycoproteins. If trimming of the

$Man_9GlcNAc_2$ oligosaccharide precursor is prevented by α1,2-mannosidases inhibitors or by gene disruption, misfolded glycoproteins are stabilized [23,24]. It suggests that *N*-glycans with untrimmed mannose residues which cannot maintain the normal functions to be synthesized or transported are accumulated and cannot be degraded, and eventually induce ER stress and activate the apoptosis pathway.

As it was reported that in preliminary clinical trials, specific inhibitors of golgi α-mannosidases II were used on the late-stage cancer patients and resulted in reduced tumor growth and metastasis with minimal side-effects [25,26]. Results in our study suggest that α-mannosidase I inhibitors also play a more important role in tumor treatment.

Acknowledgments

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