ANTICANCER RESEARCH *38*: 1353-1359 (2018)
doi:10.21873/anticanres.12358

Inhibition of Asparagine-linked Glycosylation Participates in Hypoxia-induced Down-regulation of Cell-surface MICA Expression

NAOKO YAMADA[1], NAHOKO KATO-KOGOE[2], KOJI YAMANEGI[1], HIROSHI NISHIURA[1], YUKI FUJIHARA[3], SHIGEO FUKUNISHI[3], HARUKI OKAMURA[4], NOBUYUKI TERADA[1] and KEIJI NAKASHO[1]

Departments of [1]Pathology and [3]Orthopaedic Surgery, and
[4]Laboratory of Tumor Immunology and Cell Therapy, Hyogo College of Medicine, Nishinomiya, Japan;
[2]Department of Oral Surgery, Osaka Medical College, Takatsuki, Japan

Abstract. *Background/Aim: Hypoxia down-regulates the expression of cell surface major histocompatibility class I-related chain molecule A (MICA) without increasing its shedding. Recently, the inhibition of N-linked glycosylation was also shown to reduce the cell-surface expression of MICA. We investigated the participation of asparagine (Asn)-linked glycosylation in hypoxia-induced down-regulation of cell-surface MICA using osteosarcoma cells. Materials and Methods: The cell-surface expression and Asn-N-glycosylation of MICA were estimated by flow cytometry, and western blot analyses, respectively. Results: Hypoxia reduced the expression of N-linked glycosylated MICA, as well as the ratio of N-linked glycosylated to non-glycosylated MICA. 2-Deoxy-D-glucose, which inhibits N-linked glycosylation, reduced the cell-surface expression of MICA under normoxia, while D-Mannose increased N-glycosylated MICA, increasing cell-surface MICA under hypoxia. Cells transfected with wild-type MICA expression vector expressed cell surface MICA more than those transfected with mutant MICA expression vectors designed for abrogation of N-linked glycosylation. Conclusion: The inhibition of Asn-N-linked glycosylation participates in hypoxia-induced down-regulation of cell-surface expression of MICA.*

Tumor cells express natural killer group 2 member D (NKG2D) ligands on their cell surface, that are ligands of activating receptor NKG2D that is expressed on the cell surface of cytotoxic immune cells, such as natural killer (NK), γδT- and $CD8^+$αβT-cells (1). In humans, two families of NKG2D ligands, the major histocompatibility (MHC) class I-related chain molecules A and B (MICA and MICB), and UL-16 binding protein (ULBP)-1, 2, 3 and 4 have been identified as the functional ligands for NKG2D (2). The binding of an NKG2D receptor to its ligand activates NK and γδ-T-cells and co-stimulates tumor-antigen-specific $CD8^+$αβ-T-cells (3). Thus, NKG2D ligands expressed on the cell surface of tumor cells are important for cytotoxic activity of immune cells. Therefore, proteolytic cleavage of the extracellular region of the NKG2D ligands or down-regulation of their expression on the cell surface renders tumor cells resistant to the cytotoxic activity of immune cells.

Hypoxia is a common condition found within a variety of solid tumors. Adaptive responses of tumor cells to hypoxia lead to development of malignant phenotypes which promote angiogenesis, invasion, metastasis and resistance to chemotherapy and radiotherapy (4-6). Hypoxia-inducing factor-1 (HIF1) plays a pivotal role in the adaptive responses of tumor cells to hypoxia. HIF1, which is a heterodimer with an oxygen-sensitive HIF1α subunit and a constitutively expressed HIF1β subunit, binds the hypoxia-responsive element and activates the transcription of target genes (7-9). Under normoxic conditions, HIF1α is rapidly degraded through the ubiquitin-proteasome pathway; however, under hypoxic conditions, HIF1α is stabilized and accumulates in cells (8, 10).

Regarding hypoxia-induced resistance to the cytotoxic activity of immune cells, we reported that hypoxia down-regulates the expression of cell-surface MICA in osteosarcoma cell lines without promoting the cleavage of extracellular region of MICA (11). However, the mechanisms underlying the down-regulation of cell-surface MICA remain unsolved.

Andersen *et al.* (12) and Mellergaard *et al.* (13) showed that the inhibition of *N*-glycosylation of asparagine (Asn) of MICA results in a decrease in the expression of cell-surface MICA. Therefore, in the present study, we investigated the

Correspondence to: Naoko Yamada, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan. E-mail: ynaoko@hyo-med.ac.jp

Key Words: Hypoxia, osteosarcoma, NKG2D ligand, MICA, glycosylation.

role of inhibition of *N*-linked glycosylation of MICA in hypoxia-induced down-regulation of the cell-surface expression of MICA.

Materials and Methods

Cell culture. U2-OS human osteosarcoma cells were purchased from the American Type Culture Collection (Rockville, MD, USA). HOS and SaOS-2 human osteosarcoma cells were purchased from the Riken BRC Cell Bank (Tsukuba, Ibaraki, Japan). All osteosarcoma cells were maintained in high-glucose medium: Dulbecco's modified Eagle's medium (DMEM) containing 4500 mg/l glucose (Sigma-Aldrich, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; PAA, Pasching, Austria). High-glucose medium was used for passage culture of osteosarcoma cells. However, this glucose concentration (4,500 mg/l) is much higher than the blood glucose concentration in the human body (about 1,000 mg/l). Since tumor cells use anaerobic glycolysis for ATP production under hypoxic conditions, the concentration of glucose in the medium seems to be critical for the energy metabolism of tumor cells. Therefore, in all experimental cultures in the present study, we used low-glucose medium: DMEM with 1,000 mg/l glucose (Sigma-Aldrich) containing 10% FBS. Regarding the cells cultured under normoxic condition, cells were cultured in a humidified atmosphere of 20% O_2 and 5% CO_2 in air at 37˚C. Regarding the cells cultured under hypoxic condition, cells were cultured in a humidified atmosphere of 1% O_2 and 5% CO_2 in N_2 at 37˚C using a CO_2 Multi GAS incubator (Astec, Kasuya-gun, Fukuoka, Japan).

Treatment with 2-deoxy-D-glucose (2DG) or D-Mannose. 2DG and D-Mannose, purchased from Sigma-Aldrich were dissolved in sterilized distilled water (20 M). The stock solution was diluted to the desired final concentration with medium immediately before use. Cells were cultured in medium alone for 24 h, and thereafter in medium containing either 2DG or D-Mannose for 72 h.

Transient transfections. MICA has three *N*-glycosylation sites [Asn–Xaa–Thr/Ser (NXT/S) sequences; Asn8, Asn56, and Asn102] in the extracellular region (Figure 1). The full-length MICA expression vector conjugated with the HaloTag sequence (wild-type vector) and mutant MICA expression vectors with the HaloTag sequence (M1 and M0 vectors) were purchased from KAZUSA DNA Res (Kisarazu, Chiba, Japan). Asparagine at N8 was substituted by glutamine in the M1 vector, and at N8, N56 and N102 in the M0 vector. The cells were transiently transfected with these expression vectors using the transporter (NEPA21; Nepa Gene, Ichikawa, Chiba, Japan) according to the protocol of the manufacturer. Transfected cells were cultured for 72 h in low-glucose medium under 20% O_2 conditions. The lack of any marked differences among the transfection efficiencies of three vectors was confirmed by reverse transcription polymerase chain reaction (PCR), which was performed with the specific primers for the HaloTag sequence (forward; 5'-ACATGGCCCGGGTCTGAATC-3', reverse; 5'-CTTC CTTTCGGGCTTTGTTAG-3') under established PCR conditions (10 cycles at 95˚C for 15 s, at 62˚C for 15 s and at 74˚C for 35 s) using a Bio-Rad Thermal cycler (Bio-Rad, Hercules, CA, USA).

Growth of osteosarcoma cells. Cells seeded at 1×10^3 cells per well in 96-well tissue culture plates were cultured in 100 μl of medium under either 20% or 1% O_2 conditions for 72 h. The number of viable cells in each well was estimated using a Cell counting kit-8 (Dojindo, Kumamoto, Japan).

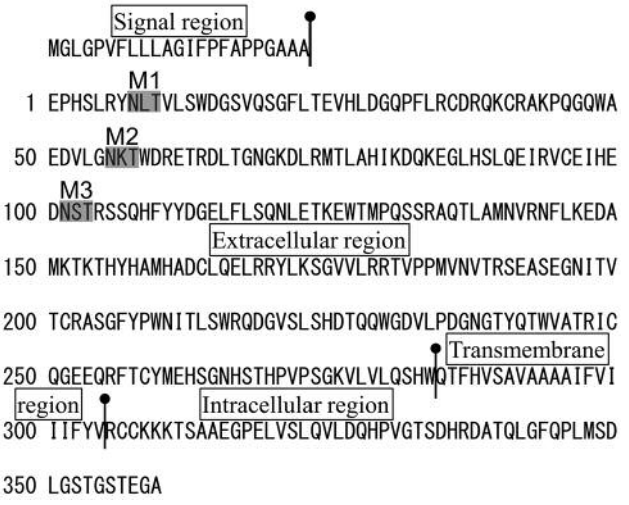

Figure 1. *Three asparagine (Asn)-N-linked glycosylation sites of MICA in the extracellular region using single letter notation for amino acids. The consensus sequence (Asn-Xaa-The/Ser: NXT/S) for N-linked glycosylation is noted by black boxes (M1, M2, M3) in the extracellular region of the mature sequence of MICA (GenBank accession number; NP_000238). The signal, extracellular, transmembrane and intracellular regions comprise -23 to -1, 1 to 284, 285 to 305 and 306 to 360 amino acids, respectively.*

Flow cytometry. Cells were harvested using brief treatment with 1 mM EDTA in phosphate-buffered saline, pH 7.4 (PBS), resuspended in the ice-cold FACS buffer (PBS containing 2% FBS) and incubated with a phycoerythrin (PE)-conjugated mouse monoclonal antibody to human MICA (monoclonal IgG antibody; 100-fold dilution; R&D Systems, Minneapolis, MN, USA) on ice for 30 min. As a control, cells were incubated with mouse IgG (100-fold dilution; R&D Systems). The cells were washed with FACS buffer, and 10,000 cells were analyzed using an LSRFortessaX-20 flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and the mean fluorescence intensity (MIF) was determined. The amount of MICA on the cell surface was expressed as the relative MIF, which was calculated by dividing the MIF value obtained on incubation with PE-labeled anti-human MICA by the MIF value obtained on incubation with mouse IgG.

To evaluate the expression of cell-surface MICA conjugated with HaloTag, cells were incubated with a mouse monoclonal antibody to HaloTag (Promega, Madison, WI, USA) on ice for 30 min. After washing cells with the FACS buffer, the cells were further incubated with a PE-labeled goat polyclonal antibody to mouse IgG (200-fold dilution; Becton, Dickinson and Company) on ice for 30 min and finally washed with FACS buffer. A total of 10,000 cells was analyzed using an LSRFortessaX-20 flow cytometer, and the MIF was determined.

Deglycosylation by treatment with peptide-N-glycosidase F (PNGase F). A PNGase F set was purchased from New England BioLabs. The PNGase F treatment was performed on homogenate from cells cultured under normoxia or hypoxia using the protocol of the manufacturer. Cell homogenate containing proteins (20 μg) in

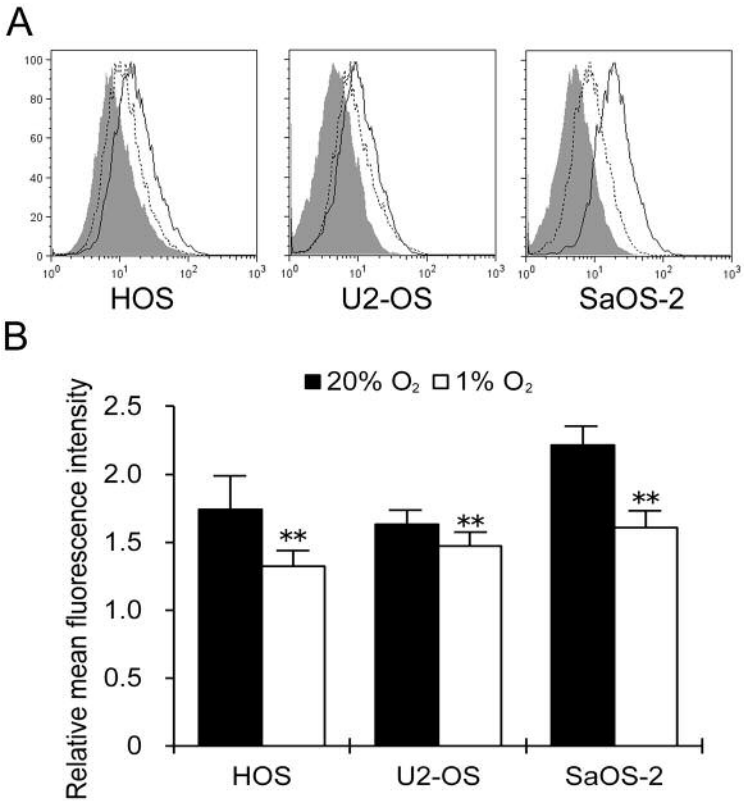

Figure 2. *Effects of hypoxia on the cell-surface expression of MICA. Three osteosarcoma cell lines were cultured for 72 h under 20% O_2 or 1% O_2 conditions. A: Representative flow cytometric profiles of cell-surface MICA. The shadow profile indicates a control profile of cells incubated with mouse IgG. The dashed and solid lines indicate the profiles of cells cultured under hypoxic and normoxic conditions, respectively. B: The mean fluorescence intensity of MICA was obtained by flow cytometry, and the relative mean fluorescence intensity of MICA was calculated as described in the Materials and Methods section. Each bar shows the mean+SD of four dishes. **Significantly different at p<0.01 from the values of the culture under 20% O_2 conditions.*

glycoprotein-denaturing buffer (0.5% SDS, 40 mM dithiothreitol) was denatured by heating at 100˚C for 10 min. Thereafter, the enzyme PNGase F, GlycoBuffer 2 (50 mM sodium acetate) and 10% NP-40 were added to the samples (1 unit PNGase F/20 μg proteins). The mixture was incubated at 37˚C for 1 h. The reaction was stopped by adding SDS loading buffer. Western blot analysis was then carried out.

Western blot analysis. The cells were homogenized with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate and a cocktail of protease inhibitors (Complete; Roche, Penzberg, Germany) using a sonicator (Sonics & Materials, Newtown, CT, USA). An aliquot of the cell homogenate containing 25 μg of proteins was boiled in a sodium dodecyl sulfate (SDS) sample buffer (New England BioLabs, Ipswich, MA, USA) and subjected to electrophoresis in a denaturing 5-10% SDS-polyacrylamide gradient gel (Atto, Tokyo, Japan). The separated proteins were transferred onto a polyvinylidene difluoride membrane (Merck Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk in PBS containing 0.1% Tween 20 and incubated with rabbit polyclonal antibody to human MICA (2000-fold dilution; GeneTex, Irvine, CA, USA), and rabbit polyclonal antibody to human β-actin (4,000-fold dilution; Thermo Fischer Scientific) at room temperature for 1 h. Proteins bound to the antibodies were detected using a horseradish peroxidase (HRP)-conjugated goat polyclonal antibody to rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA) and ECL Western Blotting Detection reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Statistical analysis. Comparisons of data of two groups were analyzed using Student's *t*-test, and data of three or more groups were analyzed using the Bonferroni multiple comparison test. A *p*-value of less than 0.05 was considered to be significant.

Results

Effects of hypoxia on cell-surface expression of MICA. HOS, U2-OS and SaOS-2 human osteosarcoma cells were cultured in medium for 72 h under normoxic or hypoxic conditions and the expression of MICA on the cell surface of these cell lines was examined by flow cytometry. Hypoxia significantly reduced the expression of MICA on the cell surface of HOS, U2-OS and SaOS-2 cells by about 24, 10 and 27% respectively (Figure 2).

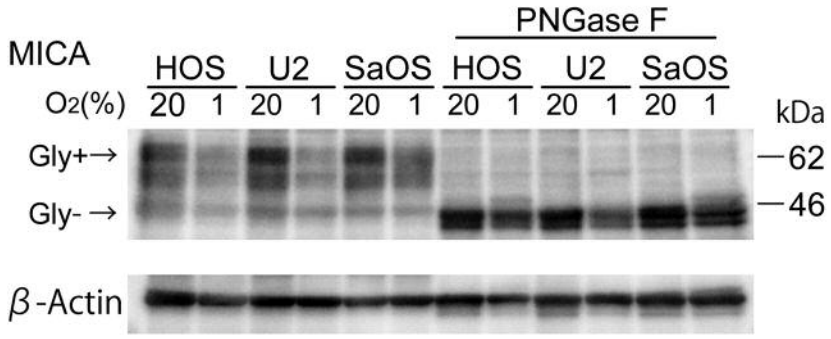

Figure 3. *Asparagine N-linked glycosylation of MICA. Three osteosarcoma cell lines were cultured for 72 h under normoxic or hypoxic conditions. Western blot analyses were carried out with cell homogenates and with cell homogenates digested by peptide-N-glycosidase F (PNGase F). PNGase F cleaves between the innermost N-acetylglucosamine and asparagine of N-linked glycosylated glycoproteins. Gly+ and Gly- indicate MICA with and without N-linked glycosylation, respectively.*

Osteosarcoma cells were cultured for 72 h under hypoxic and normoxic conditions, and then the homogenates of these cells were analyzed by western blot analyses. MICA migrates at the position of molecular weight (MW) just above 62 kDa, but western blot analyses showed another band of MICA at just below 46 kDa. Western blot analyses following the treatment of the cell homogenates with a PNGase F enzyme, which cleaves between the innermost *N*-acetylglucosamine and asparagine of *N*-linked glycosylated glycoproteins, showed that MICAs at just below 46 kDa and just above 62 kDa were MICA without Asn-*N*-linked glycosylation and Asn-*N*-linked glycosylated MICA, respectively (Figure 3). The band of Asn-*N*-linked glycosylated MICA was lighter in western blots of homogenates of cells cultured under hypoxic conditions than in blots of homogenates of cells cultured under normoxic conditions (Figure 3). In addition, the ratio of the density of the band of Asn-*N*-linked glycosylated MICA to that of MICA without Asn-*N*-linked glycosylation was less under hypoxic conditions than under normoxic conditions (Figure 3), suggesting that MICA is less Asn-*N*-linked glycosylated under hypoxic conditions than under normoxic conditions.

Hypoxia and N-glycosylation of MICA. It has been reported that 2DG inhibits *N*-linked glycosylation as well as anaerobic glycolysis (14). Therefore, we examined the effects of 2DG on the expression of cell surface MICA and Asn-*N*-linked glycosylation of MICA in osteosarcoma cells cultured under normoxic conditions for 72 h. 2DG reduced the expression of Asn-*N*-linked glycosylated MICA as well as the ratio of Asn-*N*-linked glycosylated MICA to MICA without Asn-*N*-linked glycosylation, concurrently reducing the expression of cell-surface MICA (Figure 4A and E). 2DG also reduced the number of cells dose-dependently (Figure 4B). Mannose-6-phospahte is essential for *N*-linked glycosylation, and is produced from glucose if mannose is deficient. Therefore, we examined the effects of D-Mannose on the expression of cell-surface MICA and Asn-*N*-linked glycosylation of MICA in osteosarcoma cells cultured under hypoxic conditions for 72 h. D-Mannose increased Asn-*N*-linked glycosylated MICA as well as the ratio of Asn-*N*-linked glycosylated MICA to MICA without Asn-*N*-linked glycosylation, concurrently increasing the expression of cell-surface MICA (Figure 4C and E). D-Mannose also increased the cell number dose-dependently (Figure 4D). Figure 4 also shows that the ratio of Asn-*N*-linked glycosylated MICA to MICA without Asn-*N*-linked glycosylation was less under hypoxic conditions than normoxic conditions in all three cell lines.

Asn-N-Linked glycosylation of MICA and the expression of cell-surface MICA. MICA has three Asn-*N*-glycosylation consensus sites [Asn–Xaa–Thr/Ser (NXT/S) sequences] (N8, N56, and N102) at the MHC I region in the extracellular region (Figure 1). Mellergaard *et al*. (13) have reported that Asn-*N*-linked glycosylation at the site of N8 is indispensable for the cell-surface expression of MICA. We transfected osteosarcoma cells with the full-length MICA expression vector (wild-type) and the mutant MICA expression vectors M1 and M0, in which asparagine at N8, and at N8, N56 and N102 were substituted by glutamine (Figure 1), and the expression of cell-surface MICA was examined by flow cytometry after 72-h culture under normoxic conditions. Flow cytometry showed that the expression of cell-surface MICA produced by cells with the mutant vectors M1 and M0 was reduced (Figure 5).

Discussion

2DG reduced Asn-*N*-linked glycosylated MICA as well as the ratio of Asn-*N*-linked glycosylated MICA to MICA without Asn-*N*-linked glycosylation in cells cultured under normoxic conditions, reducing the cell-surface expression of MICA concurrently. These findings were in good agreement with the study of Anderson *et al*. (12). Furthermore, cells transfected

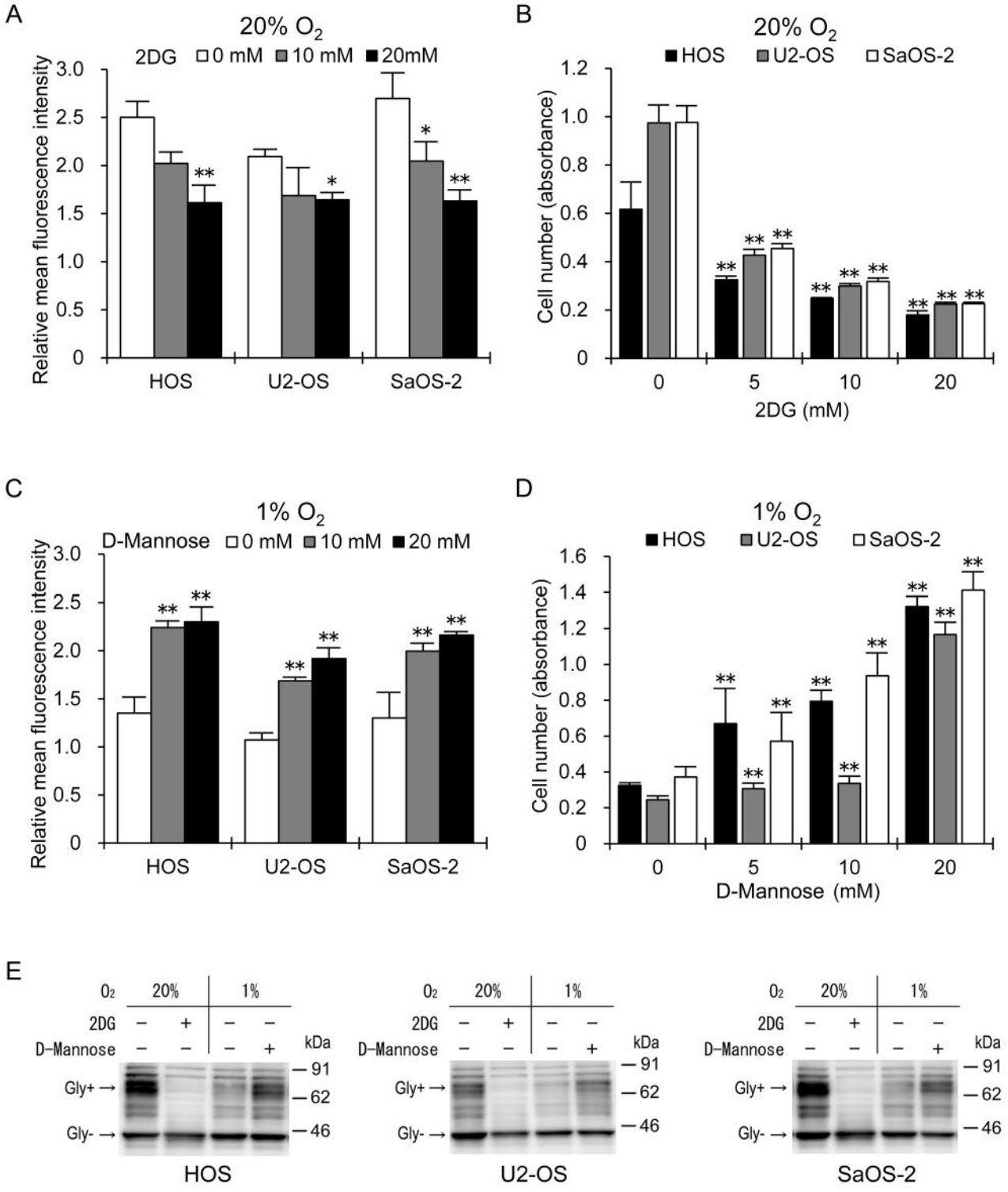

Figure 4. *Effects of 2DG and D-Mannose on the cell-surface expression of MICA, cell growth and asparagine-N-linked glycosylation. Osteosarcoma cells were cultured in medium with or without 2DG under normoxic conditions for 72 h, or with or without D-Mannose under hypoxic conditions for 72 h. The expression of cell-surface MICA represented by the relative mean fluorescence intensity was analyzed by flow cytometry (A and C), and the cell number represented by absorbance was determined (B and D). Furthermore, western blot analyses were carried out with cell homogenates (E). Gly+ and Gly- indicate MICA with and without N-linked glycosylation, respectively. Data are the mean+SD of 3-4 dishes. Significantly different at *p<0.05 and **p<0.01 from the values of cells cultured in medium without 2DG or D-Mannose.*

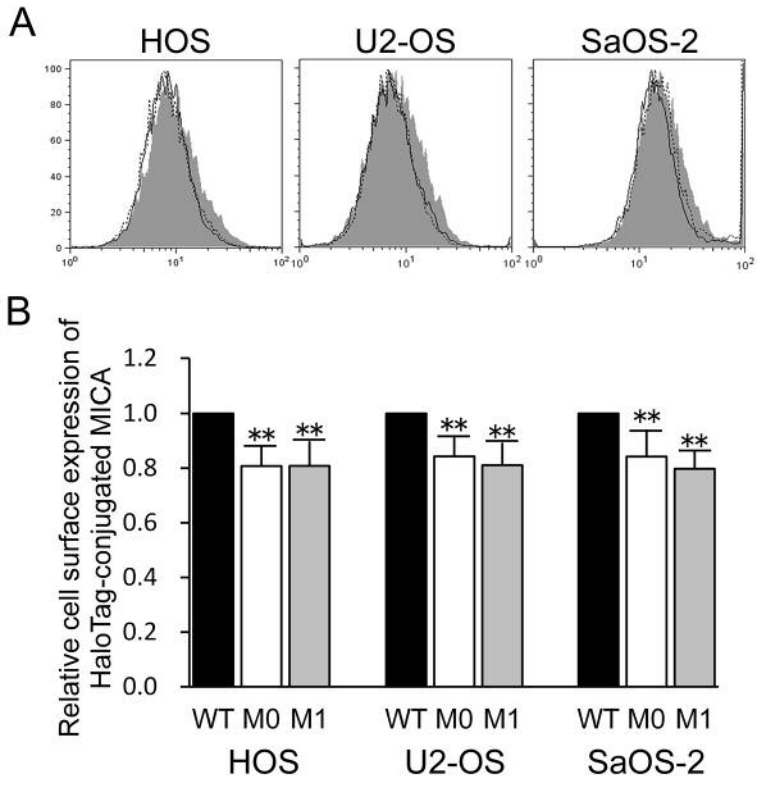

Figure 5. *Effect of asparagine (Asn)-N-linked glycosylation on the cell-surface localization of MICA. Osteosarcoma cells were transiently transfected with a plasmid encoding either HaloTag-conjugated wild-type (WT) MICA or HaloTag-conjugated mutant MICA with a Asn-N-linked glycosylation site mutation, M0 (N8Q, N56Q and N102Q) or M1 (N8Q). The cells were cultured for 72 h. A: Representative flow cytometric profiles of cell-surface HaloTag-conjugated MICA protein. The shadow profile, dashed and solid lines indicate the profiles of cells transfected with WT, M0 and M1 plasmid, respectively. B: The expression of cell surface HaloTag-conjugated wild-type or mutant MICA, represented by the relative expression, was estimated by flow cytometry. Each bar shows the mean+SD of four dishes. Significantly different at **p<0.01 from the values for WT.*

with the mutant MICA expression vectors conjugated with the HaloTag sequence, in which asparagine at N8 and at N8, N56 and N102 was substituted by glutamine, had lower cell surface expression of MICA with HaloTag than those transfected with the wild-type vector after culture under normoxic conditions. These findings agree with the study of Mellergaard *et al*. (13). In addition, D-Mannose increased the expression of MICA on the surface of cells cultured under hypoxic conditions, concurrently increasing Asn-*N*-linked glycosylated MICA as well as the ratio of Asn-*N*-linked glycosylated MICA to MICA without Asn-*N*-linked glycosylation. Taken together, these findings show that the efficient expression of cell-surface MICA in the osteosarcoma cells used in this study requires Asn-*N*-linked glycosylation of MICA.

Hypoxia reduced not only the amount of Asn-*N*-linked glycosylated MICA, but also the ratio of Asn-*N*-linked glycosylated MICA to MICA without Asn-*N*-linked glycosylation (Figures 3 and 4). These results suggest that hypoxia inhibits Asn-*N*-linked glycosylation of MICA. Therefore, it is conceivable that hypoxia inhibits Asn-*N*-linked glycosylation which participates in the down-regulation of cell-surface MICA by hypoxia. We previously showed that the knockdown of *HIF1α* mRNA using siRNA reduced HIF1α

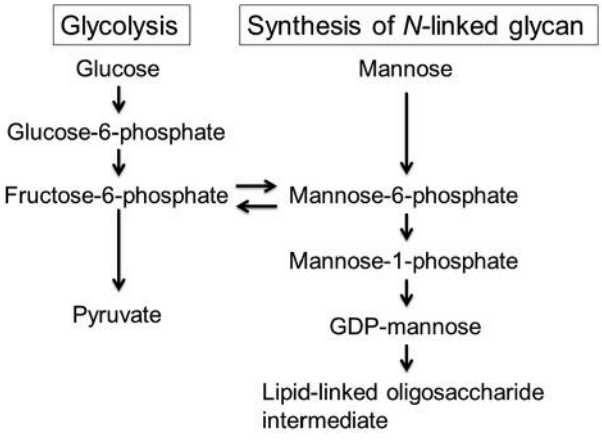

Figure 6. *Relationship between anaerobic glycolysis and the synthesis of N-linked glycan.*

accumulation in osteosarcoma cells cultured under hypoxic conditions, concomitantly increasing *MICA* mRNA and the expression of cell-surface MICA (11). Therefore, not only the inhibition of Asn-*N*-linked glycosylation but also HIF1α-induced inhibition of MICA synthesis is likely to be involved in hypoxia-induced down-regulation of cell-surface MICA.

Under hypoxic conditions, tumor cells seem to be deficient in intracellular glucose, as glucose is used in anaerobic glycolysis for ATP production under anaerobic conditions. Mannose-6-phosphate is essential for the formation of *N*-linked glycan and is obtained from fructose-6-phosphate, a metabolite of anaerobic glycolysis (Figure 6). Therefore, the inhibition of Asn-*N*-linked glycosylation of MICA under hypoxic conditions seems to be partly ascribed to deficiency in mannose-6-phosphate due to the hypoxia-induced shortage of intracellular glucose. Consistent with this speculation, we showed that D-Mannose increased Asn-*N*-linked glycosylated MICA in cells cultured under hypoxic conditions. Furthermore, supporting this speculation, several studies have reported that even under aerobic circumstances, glucose starvation results in production of abnormal *N*-linked glycan (15,16) and that D-Mannose abrogates this abnormality (15-19).

D-Mannose dose-dependently increased the cell number during culture under hypoxic conditions. Since D-Mannose can be converted to fructose-6 phosphate, a metabolite in anaerobic glycolysis, and used for ATP production, the effect of D-Mannose on the cell number seems to be the prevention of cell death due to the shortage of ATP. In contrast, 2DG reduced the cell number in culture under normoxic conditions. It has been reported that 2DG exerts a toxic effect through inhibition of *N*-linked glycosylation rather than glycolysis under anaerobic conditions, although 2DG can inhibit glycolysis (14).

In conclusion, the present study suggests that hypoxia inhibits Asn-*N*-linked glycosylation of MICA, which participates in the down-regulation of cell-surface MICA by hypoxia.

Acknowledgements

This work was supported by Grant-in-Aid for Researchers, Hyogo College of Medicine, 2014.

References

1 Raulet DH: Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol *3(10)*: 781-790, 2003.
2 Nausch N and Cerwenka A: NKG2D ligands in tumor immunity. Oncogene *27(45)*: 5944-5958, 2008.
3 Salih HR, Antropius H, Gieseke F, Lutz SZ, Kanz L, Rammensee HG and Steinle A: Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. Blood *102(4)*: 1389-1396, 2003.
4 Wilson WR and Hay MP: Targeting hypoxia in cancer therapy. Nat Rev Cancer *11(6)*: 394-410, 2011.
5 Chouaib S, Messai Y, Couve S, Escudier B, Hasmim M and Noman MZ: Hypoxia promotes tumor growth in linking angiogenesis to immune escape. Front Immunol *3*: 21, 2012.
6 Tang CM and Yu J: Hypoxia-inducible factor-1 as a therapeutic target in cancer. J Gastroenterol Hepatol *28(3)*: 401-405, 2013.
7 Semenza GL: Physiology meets biophysics: visualizing the interaction of hypoxia-inducible factor 1 alpha with p300 and CBP. Proc Natl Acad Sci USA *99(18)*: 11570-11572, 2002.
8 Semenza GL: Angiogenesis in ischemic and neoplastic disorders. Annu Rev Med *54*: 17-28, 2003.
9 Melillo G: Inhibiting hypoxia-inducible factor 1 for cancer therapy. Mol Cancer Res *4(9)*: 601-605, 2006.
10 Giaccia A, Siim BG and Johnson RS: HIF1 as a target for drug development. Nat Rev Drug Discov *2(10)*: 803-811, 2003.
11 Yamada N, Yamanegi K, Ohyama H, Hata M, Nakasho K, Futani H, Okamura H and Terada N: Hypoxia downregulates the expression of cell surface MICA without increasing soluble MICA in osteosarcoma cells in a HIF1α-dependent manner. Int J Oncol *41(6)*: 2005-2012, 2012.
12 Andresen L, Skovbakke SL, Persson G, Hagemann-Jensen M, and Hansen KA, Jensen H and Skov S: 2-Deoxy D-glucose prevents cell surface expression of NKG2D ligands through inhibition of *N*-linked glycosylation. J Immunol *188(4)*: 1847-1855, 2012.
13 Mellergaard M, Skovbakke SL, Schneider CL, Lauridsen F, Andresen L, Jensen H and Skov S: *N*-Glycosylation of asparagine 8 regulates surface expression of major histocompatibility complex class I chain-related protein A (MICA) alleles dependent on threonine 24. J Biol Chem *289(29)*: 20078-20091, 2014.
14 Kurtoglu M, Gao N, Shang J, Maher JC, Lehrman MA, Wangpaichitr M, Savaraj N, Lane AN and Lampidis TJ: Under normoxia, 2-deoxy-D-glucose elicits cell death in select tumor types not by inhibition of glycolysis but by interfering with *N*-linked glycosylation. Mol Cancer Ther *6(11)*: 3049-3058, 2007.
15 Gershman H and Robbins PW: Transitory effects of glucose starvation on the synthesis of dolichol-linked oligosaccharides in mammalian cells. J Biol Chem *256(15)*: 7774-7780, 1981.
16 Rearick JI, Chapman A and Kornfeld S: Glucose starvation alters lipid-linked oligosaccharide biosynthesis in Chinese hamster ovary cells. J Biol Chem *256(12)*: 6255-6261, 1981.
17 Turco SJ: Modification of oligosaccharide-lipid synthesis and protein glycosylation in glucose-deprived cells. Arch Biochem Biophys *205(2)*: 330-339, 1980.
18 Datema R and Schwarz RT: Effect of energy depletion on the glycosylation of a viral glycoprotein. J Biol Chem *256(21)*: 11191-11198, 1981.
19 Panneerselvam K and Freeze HH: Mannose corrects altered *N*-glycosylation in carbohydrate-deficient glycoprotein syndrome fibroblasts. J Clin Invest *97(6)*: 1478-1487, 1996.

Received December 28, 2017
Revised January 24, 2018
Accepted January 31, 2018