Overexpression of human epidermal growth factor receptor 2 (HER2) has been reported in glioblastoma as well as breast, gastric, lung, colorectal, and pancreatic cancers. Its expression is associated with poor clinical outcomes. Anti-HER2 antibodies have provided significant survival benefits to patients with HER2-overexpressing breast and gastric cancers. We recently developed an anti-HER2 monoclonal antibody (mAb), H2Mab-19 (IgG2b, kappa), by immunizing mice with the extracellular domain of HER2, which is expressed in LN229 glioblastoma cells. In this study, we investigated the antitumor activity of H2Mab-19 in an LN229 glioblastoma xenograft model. H2Mab-19 showed high binding affinity (K_D: 1.1 × 10^{-8} M) against LN229 cells. Furthermore, H2Mab-19 significantly reduced tumor development in an LN229 xenograft. These results suggest that treatment with H2Mab-19 may be a useful therapy for patients with HER2-expressing glioblastomas.

Keywords: HER2, monoclonal antibody, antitumor activity, glioblastoma

Introduction

Gliomas represent the most common primary brain tumor, and glioblastoma multiforme is the most frequent and malignant type of glioma.\textsuperscript{(1)} Despite advances in surgical techniques, radiation therapy, and adjuvant chemotherapy, their prognoses remain poor. Many antigens, including epidermal growth factor receptor (EGFRwt), its glioma-associated deletion variant EGFRvIII, tenascin, chondroitin sulfate proteoglycans, and lacto-series gangliosides, have been found in gliomas, and upregulation of those molecules has been observed in brain tumor cells.\textsuperscript{(2)} Although these molecules are under investigation as therapeutic targets, multiple entities may ultimately have to be targeted to compensate for tumor heterogeneity. Human epidermal growth factor receptor 2 (HER2) has also been reported to be expressed in glioblastomas, and represents one of the molecular targets for immunotherapy.\textsuperscript{(3–8)}

A novel anti-HER2 monoclonal antibody (mAb) (H2Mab-19; IgG2b, kappa) was recently developed by immunizing mice with the purified recombinant ectodomain of HER2.\textsuperscript{(9)} H2Mab-19 exerts antitumor activity in BT-474, HSC-2, and SAS xenografts by antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), suggesting that treatment with H2Mab-19 may be a useful therapy for patients with HER2-expressing breast and oral cancers. In this study, we investigated whether H2Mab-19 exhibits antitumor activity in a mouse xenograft model of glioblastoma.
Alexa Fluor 488-conjugated antimouse IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA). Fluorescence data were collected using an EC800 Cell Analyzer (Sony Corp., Tokyo, Japan).

**Determination of the binding affinity**

LN229 cells were suspended in 100 µL serially diluted H2Mab-19 (6 ng/mL–100 µg/mL), followed by the addition of Alexa Fluor 488-conjugated antimouse IgG (1:200; Cell Signaling Technology, Inc.). Fluorescence data were collected using an EC800 Cell Analyzer (Sony Corp.). The dissociation constant ($K_D$) was obtained by fitting binding isotherms to built-in one-site binding models in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA).

**Antitumor activity of H2Mab-19 in the xenografts of glioblastoma**

Sixteen 6-week-old female BALB/c nude mice were purchased from Charles River Laboratories and used at 7 weeks of age. All animal experiments were performed in accordance with relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. Animal studies for antitumor activity were approved by the institutional committee for experiments of the Institute of Microbial Chemistry (Permit number: 2019-021). Mice were monitored for health and weight every 2 to 4 days. The duration of the experiments was 3 weeks. A bodyweight loss exceeding 25% and a maximum tumor size exceeding 3000 mm$^3$ were designated as humane endpoints. Mice were euthanized by cervical dislocation and death was verified by respiratory arrest and cardiac arrest.

LN229 cells (0.3 mL of $1.33 \times 10^8$ cells/mL in DMEM) were mixed with 0.5 mL BD Matrigel Matrix Growth Factor Reduced (BD Biosciences, San Jose, CA). One hundred microliters of this suspension ($5 \times 10^6$ cells) was injected subcutaneously into the left flank. After day 1, 100 µL of H2Mab-19 and control mouse IgG (Sigma-Aldrich Corp.) in 100 µL of PBS were injected intraperitoneal (i.p.) into treated and control mice, respectively. Additional antibodies were

FIG. 1. Characterization of H2Mab-19 using flow cytometry. (A) LN229 cells were treated with H2Mab-19. The black line represents the negative control (blocking buffer). (B) Determination of the binding affinity of H2Mab-19 for LN229 cells using flow cytometry. H2Mab-19, anti-human epidermal growth factor receptor 2 monoclonal antibody.
then injected on days 6 and 14. Twenty days after cell implantation, all mice were euthanized by cervical dislocation and tumor diameters and volumes were determined as previously described.\(^{(11)}\)

Statistical analyses

All data are expressed as mean±standard error of the mean. Statistical analysis used analysis of variance and Tukey–Kramer’s test with R statistical. \(p\) values <0.05 were considered statistically significant.

Results

Characterization of H2Mab-19 against a glioblastoma cell line

We first measured the surface expression of HER2 in the LN229 human glioblastoma and HER2-overexpressing LN229/HER2 cell lines. As expected, H2Mab-19 recognized endogenous HER2 in LN229 cells and overexpressed HER2 of LN229/HER2 cells by flow cytometry (Fig. 1A).

The binding affinity (\(K_D\)) of H2Mab-19 to LN229 cells was \(1.1 \times 10^{-8}\) M (Fig. 1B), indicating that H2Mab-19 exhibits a

FIG. 2. Evaluation of the antitumor activity of H2Mab-19. (A) Tumor volume was measured in LN229 xenografts. (B) Resected tumors of LN229 xenografts (day 20). (C) Tumor weight was measured in LN229 xenografts (day 20). (D) Appearance of treated mice on day 20. (E) Body weights of the mice with the LN229 xenografts were measured for 20 days. Values represent the mean±SEM. **\(p < 0.01\), *\(p < 0.05\), the Tukey–Kramer’s test. n.s.: not significant. SEM, standard error of the mean.
high affinity for HER2-expressing glioblastoma cell lines. These results suggest the possibility of targeting HER2 as an antigen for immunotherapy.

**Antitumor activity of H2Mab-19 in mouse xenografts of glioblastoma**

Next, we investigated whether H2Mab-19 induces cytotoxic activity against glioblastoma in vivo. To study the antitumor activity of H2Mab-19 on cell growth in vivo, LN229 cells were implanted subcutaneously in the flanks of nude mice. H2Mab-19 and control mouse IgG were injected i.p. three times (days 1, 6, and 14 after cell injection) into treated and control mice, respectively. Tumor formation was observed in both the H2Mab-19-treated and control groups. H2Mab-19 treatment significantly reduced tumor development compared with development in control mice on day 10 (p < 0.05), day 14 (p < 0.01), day 17 (p < 0.01), and day 20 (p < 0.01; Fig. 2A). Resected tumors are depicted in Figure 2B. Tumor weight from H2Mab-19-treated mice was significantly less than that of the IgG-treated control mice (p < 0.05; Fig. 2C). Mice on day 20 are depicted in Figure 2D. Total body weight was not significantly different between the two groups (Fig. 2E). These results indicate that H2Mab-19 exerts a significant antitumor effect against HER2-expressing glioblastomas.

**Discussion**

HER2 overexpression has been reported in breast and gastric cancers, and is associated with poor clinical outcomes. The humanized anti-HER2 mAbs, trastuzumab and pertuzumab, have been used in the treatment of HER2-positive breast cancer. Treatment with trastuzumab resulted in significant survival benefits in these patients. The combination of trastuzumab and pertuzumab with chemotherapy has led to significant improvements in overall survival. Recently, trastuzumab deruxtecan (DS-8201), which consists of three components, a novel enzymecleavable linker, and a topoisomerase I inhibitor, exerts antitumor activity even in low-HER2-expressing tumors. Therefore, we still require additional sensitive and specific anti-HER2 mAbs, the epitope of which is different than trastuzumab and pertuzumab. We have tried to develop many anti-HER2 mAbs that react with not only breast cancers, but also colon cancers, pancreatic cancers, and glioblastoma using CasMab technology.

We previously developed anti-HER2 mAbs, H2Mab-77 and H2Mab-139. These antibodies are useful for flow cytometry, Western blot, and immunohistochemical analyses. The subclass of these mAbs was determined to be mouse IgG1. Therefore, they do not possess ADCC or CDC activities. We further tried to develop an anti-HER2 mAb of the IgG2b subclass using CasMab technology, because mouse IgG2b antibodies exhibit ADCC and CDC activity. A novel anti-HER2 mAb (H2Mab-19) of IgG2b was recently established, which exhibited antitumor activity in breast or oral cancer xenografts by ADCC and CDC. In this study, we investigated whether H2Mab-19 exhibits antitumor activities in LN229 mouse xenograft models, since HER2 has been reported to be expressed in glioblastomas.

Antigen–antibody affinity is thought to be an important factor influencing the outcome of antibody-based therapy. The binding affinity (Kd) of H2Mab-19 to LN229 was determined to be 1.1 x 10^-9 M using flow cytometry, indicating that H2Mab-19 shows high affinity to HER2-expressing glioblastoma cell lines. This is similar to the Kd of H2Mab-19 to BT-474 (2.3 x 10^-9 M), HSC-2 (9.5 x 10^-9 M), and SAS (5.5 x 10^-9 M).

We selected an LN229 cell line for the in vivo study, since LN229 is useful for glioblastoma xenograft models. In our previous study, H2Mab-19 treatment significantly reduced tumor development in breast and oral cancer xenograft models. H2Mab-19 also exerted antitumor activity against LN229 xenografts in this study (Fig. 2). Further studies using other glioblastoma xenografts should be performed to confirm that H2Mab-19 could represent a viable therapy for patients with HER2-expressing glioblastomas.

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**Author Disclosure Statement**

No competing financial interests exist.

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