Melanoma is a life-threatening disorder and its incidence is increasing gradually. Despite the numerous treatments approaches, conventional systemic chemotherapy has not reduced the mortality rate among melanoma patients, probably due to the induction of toxicity to normal tissues. Recently, we have developed folate-conjugated methyl-β-cyclodextrin (FA-M-β-CyD) and clarified its potential as a new antitumor agent involved in autophagic cell death. However, it remains uncertain whether FA-M-β-CyD exerts antitumor effects against melanomas. Therefore, in this study, we investigated the effects of FA-M-β-CyD on the folate receptor-α (FR-α)-expressing melanoma cell-selective cytotoxic effect. FA-M-β-CyD showed cytotoxic effects in Ihara cells, a human melanoma cell line expressing FR-α. In sharp contrast to methyl-β-cyclodextrin, FA-M-β-CyD entered Ihara cells [FR-α(+) ] through FR-α-mediated endocytosis. Additionally, FA-M-β-CyD elicited the formation of autophagosomes in Ihara cells. Notably, FA-M-β-CyD suppressed melanoma growth in BALB/c nude recombinase-activating gene-2 (Rag-2)/Janus kinase 3 (Jak3) double deficient mice bearing Ihara cells. Therefore, these results suggest that FA-M-β-CyD could be utilized as a potent antitumor agent for melanoma chemotherapy by regulating autophagy.

Key words  folate receptor; methyl-β-cyclodextrin; melanoma; autophagy

Melanoma remains a cause of death despite the numerous treatments approaches such as chemotherapy, immunotherapy and radiotherapy. The main risk factor of melanoma is excessive sun exposure in childhood. In patients with melanomas, an extirpation of primary melanomas should be implemented, whereas a metastasis control by chemotherapy is dedicated. However, all conventional combinations at present used in metastasis melanomas can not undoubtedly showed an enough efficacy.

To obtain the maximum treatment efficacy in melanoma chemotherapy, the drug delivery technique is extremely essential. To confer an active targeting-ability, the chemical modification of tumor-specific ligands to a drug carrier is known. Of various tumor-targeting ligands, folic acid (FA) has emerged as a remarkable targeting ligand capable of potent interaction with cancer cells expressing the folate receptor-α (FR-α) with high affinity ($K_d$: $10^{-9} - 10^{-10} \text{M}$). The expression of FR-α is limited to normal differentiated epithelial cells and is drastically elevated in malignant tissues. Therefore, FR-α is one of the potent candidate for not only a promising marker but also a target protein for therapy of melanoma.

Cyclodextrins (CyDs) are cyclic oligosaccharides and are known to be host molecules. CyDs can include various hydrophobic compounds in the cavity in solution, and often used as solubilizers against lipophilic drugs in the pharmaceutical fields. Meanwhile, CyDs at higher concentration induce hemolysis and hamper the integrity of the mucosal epithelial cells, and extract cholesterol, phospholipids and proteins from biological membranes, which are expedient for studying the function of lipid microdomains in the biological fields. In addition, methyl-β-cyclodextrin (M-β-CyD) is often used to disrupt lipid microdomains because of its ability to decrease cholesterol stores on cell membranes. Numerous studies have also demonstrated that the disruption of lipid microdomains by M-β-CyD can detriment cancer cells and trigger cell-death. Notably, Grosse et al. demonstrated that an intraperitoneal administration of M-β-CyD significantly suppressed tumor growth in tumor-bearing mice. However, the antitumor effect of M-β-CyD has poor tumor cell-selectivity.

Recently, to make an attempt to give a tumor-specific cytotoxic reaction to M-β-CyD, we previously prepared FA-conjugated M-β-CyD (FA-M-β-CyD), and evaluated its antitumor effect. FA-M-β-CyD provided great antitumor effect, compared to M-β-CyD in FR-α-expressing KB cells, a human oral squamous carcinoma cell line. The single subcutaneous injection of FA-M-β-CyD dramatically impeded the tumor growth in Colon-26 cells (FR-α(+) )-bearing mice. Additionally, the antitumor effect of FA-M-β-CyD was superior to that of doxorubicin after a subcutaneous administration, at the same dose. However, it remains uncertain whether FA-M-β-CyD provides antitumor effect for melanomas. Therefore, in this study, we investigated whether FA-M-β-CyD provides the FR-α-expressing melanoma cell-selective antitumor effect in vitro and in vivo. As a result, FA-M-β-CyD was found to induce cytotoxic effect in Ihara cells, a human melanoma cell line expressing FR-α, and promoted autophagosome formation in vitro, indicating the involvement of autophagy in the cytotoxic effect. Moreover, FA-M-β-CyD suppressed the tumor growth in mice inoculated Ihara cells. Consequently, FA-M-β-CyD
could be utilized as a novel anticancer drug through regulating autophagy for melanoma chemotherapy.

MATERIALS AND METHODS

Materials  FA and M-β-CyD were obtained from Nacalai Tesque (Kyoto, Japan) and Junsei Chemical (Tokyo, Japan), respectively. Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Nichirei (Tokyo, Japan) and Nissui Pharmaceuticals (Tokyo, Japan), respectively. Tetramethylrhodamine isothiocyanate (TRITC) was purchased from Funakoshi (Tokyo, Japan).

Synthesis of FA-M-β-CyD  FA-M-β-CyD was prepared as previously reported. Briefly, after tosylation of M-β-CyD, amino-M-β-CyD was obtained by stirred for 24h at 40°C in 25% ammonia water. FA was introduced to amino-M-β-CyD through condensation reaction, then FA-M-β-CyD was obtained.

Cell Culture and in Vitro Cytotoxic Effect  Ihara cells, a human melanoma cell line, were cultured as reported previously. The in vitro antitumor effect was performed by the WST-1 method, as reported previously. Briefly, Ihara cells (5×10⁴/96-well microplate) were treated with 10 mM M-β-CyD, amino-M-β-CyD was obtained by stirred for 24h at 40°C in 25% ammonia water. FA was introduced to amino-M-β-CyD through condensation reaction, then FA-M-β-CyD was obtained.

Cellular Association of TRITC-FA-M-β-CyD  Ihara cells (1×10⁶/35 mm dish) were incubated with 1 mL of DMEM containing TRITC-FA-M-β-CyD (10 µM) at 37°C for 1 h. Hoechst 33342 (10 µg/mL) was incubated at 37°C for 10 min. After washing with PBS (pH 7.4), the culture medium was added. A fluorescence microscope (KEYENCE Biozero BZ-8000) was used for the detection of TRITC and Hoechst33342.

Autophagosome Formation  Ihara cells (1×10⁶/35 mm dish) were treated with FA-M-β-CyD (5 µM) for 2 h, in the absence and presence of pretreatment with FA for 4h, and then the cells were incubated with Cyto-ID® Autophagy Detection Kit (Enzo Life Sciences, Farmingdale, NY, U.S.A.). A fluorescence microscope of Biozero BZ-8000 (KEYENCE) was used for cell observation.

In Vivo Antitumor Effect of FA-M-β-CyD in Nude Retrains-Activating Gene-2 (Rag-2)/Janus Kinase 3 (Jak3) KO Mice Bearing Ihara Cells  Four-weeks-old BALB/c nude Rag-2/Jak3 KO mice were subcutaneously injected the suspension containing Ihara cells (7.5×10⁶ cells/100 µL), FR-α expressing cells. About 7 d later, the mannitol solution (5% (w/v)) dissolved with M-β-CyD (10 mg/kg) or FA-M-β-CyD (10 mg/kg) was administered by the single subcutaneous injection to BALB/c nude Rag-2/Jak3 KO mice bearing Ihara cells. The tumor volumes were determined by the equation (volume=LW²/2), where L is the longest dimension parallel to the skin surface and W is the dimension perpendicular to L and parallel to the surface. Animal experiments were approved by the Ethics Committee for Animal Care and Use of Kumamoto University.

Statistics  All experiments were performed in triplicate in each series of measurements, and each series was repeated more than three times. The experimental results are shown as mean±standard error of the mean (S.E.M.). Significance levels for comparisons between samples were determined with Scheffe’s test. The level of statistical significance was set at p<0.05.
RESULTS AND DISCUSSION

Cytotoxic Effect of FA-M-β-CyD  To reveal the anti-tumor effect of FA-M-β-CyD in melanoma cells, we investigated cytotoxic effect of FA-M-β-CyD in Ihara cells, a human melanoma cell line which highly expresses FR-α. M-β-CyD and FA-M-β-CyD showed great cytotoxic effects in Ihara cells, compared to control (Figs. 1A, B). There was no significant difference of the cytotoxic effect of M-β-CyD in the absence and presence of FA as a competitor of FR in Ihara cells (Fig. 1A). In sharp contrast, the cytotoxic effect of FA-M-β-CyD in Ihara cells was drastically inhibited by the addition of FA (Fig. 1B). These results indicate the cytotoxic effect of FA-M-β-CyD mediated by FR-α in Ihara cells.

Cellular Association and Intracellular Distribution of FA-M-β-CyD  To obtain the detail of the mechanism for the FR-α-mediated cytotoxic effect of FA-M-β-CyD, we studied whether TRITC-FA-M-β-CyD is associated with Ihara cells (Fig. 2). Strikingly, TRITC-FA-M-β-CyD was highly associated with Ihara cells, despite CyDs are known to be biomembrane-impermeable. Furthermore, the association of TRITC-FA-M-β-CyD was inhibited by the addition of FA as a competitor of FR. These results suggest that FA-M-β-CyD could be associated with Ihara cells through FR-α.

Next, we investigated the intracellular distribution of TRITC-FA-M-β-CyD in Ihara cells (Fig. 3). The intracellular distribution of TRITC-M-β-CyD was almost negligible after 1h treatment. Meanwhile, cellular uptake of TRITC-FA-M-β-CyD in Ihara cells was observed. In addition, internalization of TRITC-FA-M-β-CyD into Ihara cells was significantly inhibited by the addition of FA. Herein, FR-α has been reported to be endocytosed via clathrin-independent carrier/GPI-anchored proteins enriched early endosomal compartment (CLIC/GEEC).29) Taken together, these results indicate that FA-M-β-CyD distributed in Ihara cells via CLIC/GEEC endocytosis after the recognition by FR-α and provided potent cytotoxic effects.

Involvement of Autophagy in Cytotoxic Effect by FA-M-β-CyD  Autophagy, the major lysosomal pathway for recycling intracellular components including organelles, is emerging as a key process regulating tumorigenesis and cancer therapy.30–34) The dynamic roles for autophagy in cancer are the tumor suppressive effect at the early stage of cancer development, but are the growth effect at the advanced stage of tumors. Likewise, the stimulation of autophagy in response to therapeutics can contextually favor or weaken chemoresistance and antitumor immunity. Therefore, the understanding whether and how autophagy can be harnessed to kill cancer cells is essential for cancer chemotherapy. There are sev-
whether autophagosome formation in Ihara cells is elicited by FA-M-β-CyD, using Cyto-ID® Autophagy Detection Kit, which detects autophagic vacuoles in cells. As shown in Fig. 4, the expression of autophagosomes in Ihara cells was induced after treatment with FA-M-β-CyD for 2 h. These results suggest that FA-M-β-CyD induces the formation of autophagosomes in Ihara cells.

Next, we examined the effects of autophagy inhibitors such as bafilomycin A1 and chloroquine on cell viability of Ihara cells after treatment with FA-M-β-CyD. Herein, bafilomycin A1 and chloroquine prevent endosomal acidification, which leads to an inhibition of both fusion of autophagosomes with lysosome and lysosomal protein degradation. The cell viabilities of Ihara cells treated with FA-M-β-CyD in the presence of bafilomycin A1 and chloroquine were higher than that with FA-M-β-CyD alone (Fig. 5). Therefore, these data indicate that FA-M-β-CyD is likely to cause autophagic cell-death. The numerous signaling pathways related to autophagy such as mammalian target of rapamycin (mTOR) and phosphoinositide 3-kinase (PI3K)-Akt, that are commonly dysregulated in human cancer. However, it still remains vague the effects of FA-M-β-CyD on mTOR or class III PI3K-Akt function, which elicits autophagosome formation. Therefore, the further studies on the mechanism of autophagy caused by FA-M-β-CyD are required.

Antitumor Effect of FA-M-β-CyD in BALB/c Nude Rag-2/Jak3 KO Mice Bearing Ihara Cells To investigate the antitumor effect of FA-M-β-CyD in vivo, we injected FA-M-β-CyD solution subcutaneously to BALB/c nude Rag-2/Jak3 KO mice bearing Ihara cells. Here, we used BALB/c nude Rag-2/Jak3 KO mice, because these mice showed a lack of mature T and B lymphocytes and natural killer (NK) cells. As shown in Fig. 6, a subcutaneous injection of M-β-CyD slightly suppressed the tumor growth. Remarkably, FA-M-β-CyD potently tended to inhibit the tumor growth after subcutaneous injection (Fig. 6A). Furthermore, 21 d after subcutaneous injection, FA-M-β-CyD, suppressed the growth of tumor inoculated subcutaneously, compared to mannitol and M-β-CyD (Fig. 6B). Additionally, there was no significant difference in the body weight of mice between mannitol, M-β-CyD and FA-M-β-CyD systems, suggesting that FA-M-β-CyD does not have obvious side effects (Fig. 6C). In future, to obtain the maximum treatment efficacy of FA-M-β-CyD, the analysis of biodistribution of FA-M-β-CyD and the optimization studies of dose and administration timing are necessary. These results suggest that FA-M-β-CyD has the potential as a novel antitumor agent after subcutaneous injection to mice bearing melanoma.

In the present study, FA-M-β-CyD was found to induce autophagosome formation in Ihara cells, FR-α-positive cells (Figs. 1, 4, 5). Taking into the consideration of our in vivo results, FA-M-β-CyD can be applied as a novel anticancer drug through regulating autophagy for cancer chemotheraphy against FR-α-overexpressing melanoma. However, autophagy has been referred to as a double-edged sword, because excessive or sustained autophagy in tumor cells may be pro-death, particularly in apoptosis-defective cells. To understand a role of autophagy in chemotherapy is totally critical, because many anticancer drugs activated autophagy, although the consequences of autophagy activation are unclear. Therefore, further analyses of autophagic signaling elicited by FA-M-β-CyD are required.

With respect to the mechanism of FR-α-mediated endocytosis, it remains unclear the involvement of FR-α in the transport of FA-M-β-CyD in normal melanocytes and the differences between normal and malignant melanocytes. Although Sánchez-del-Campo et al. revealed that the expression
of FR-α was drastically elevated in melanoma cells, compared to normal melanocytes, suggesting the physiological and oncological importance of these findings should be further investigated.

In conclusion, in this study, we revealed the involvement of autophagy in the FR-α-expressing melanoma cell-selective antitumor effect of FA-M-β-CyD. These findings will give great information of FA-M-β-CyD as a novel autophagy inducer for melanoma chemotherapy. In future, further application of FA-M-β-CyD for the treatment of metastatic melanoma is expected.

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Conflict of Interest The authors declare no conflict of interest.

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