Response of brain specific microenvironment to P-glycoprotein inhibitor: An important factor determining therapeutic effect of P-glycoprotein inhibitor on brain metastatic tumors

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Abstract. P-glycoprotein (P-gp), a factor responsible for the multidrug resistance of tumors, is specifically expressed in brain microenvironment. To test its roles in brain metastatic tumor chemoresistance, we implanted the paclitaxel-sensitive melanoma cell line, K1735, into the skin or brain of mice and examined its paclitaxel resistances. When implanted into the skin, paclitaxel inhibited tumor growth, however, it had no inhibitory effect on cells implanted into the brain. The paclitaxel resistance of the brain K1735 tumors was eliminated by combined treatment with a P-gp inhibitor, HM30181A, and paclitaxel. Previously we found that there is a defined therapeutic window for combined treatment of brain tumors with HM30181A and paclitaxel. To determine whether it is due to responses of the brain microenvironment we measured changes in P-gp expression and function of brain endothelial cells in response to HM30181A treatment *in vitro* and *in vivo*. They were significantly increased by high-dose HM30181A treatment and it was related with the therapeutic effect loss of high-dose HM30181A treatment. Therefore, P-gp in the brain microenvironment has crucial roles in the brain metastatic tumor chemoresistance and brain microenvironment responses to P-gp inhibitor treatment should be considered in the development of brain endothelial cell-targeted chemotherapy using P-gp inhibitor.

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Introduction

Blood-born brain metastases are common in cancer patients with an incidence rate of 12-35% (1,2). Solitary brain metastasis can be managed by local treatment modalities such as irradiation or surgery. However, many patients with brain metastasis harbor two or more metastases and treatment of extensively metastasized brain tumors often has poor outcome due to resistance to conventional chemotherapy. This chemoresistance is often observed even though the primary tumors are chemosensitive (3). Therefore, it can be postulated that brain specific microenvironment influences the chemoresistance of brain metastatic tumors.

The anatomical tightness of the blood-brain barrier (BBB) causes poor paracellular penetration of anticancer drugs (4) and P-glycoprotein (P-gp) (5-7) on the luminal side of plasma membrane in brain endothelial cells (8,9) prevents its substrates from entering into the brain parenchyma through brain endothelial cells (10-12). Because many anticancer drugs are substrates of P-gp, in combination with the BBB, P-gp can produce a special microenvironment, an anticancer agent free space (13). Tumor cells use the normal brain vasculature at the early stages of metastasis, rather than making new vessels (14), therefore, brain specific microenvironment would be critical in the chemosensitivity of brain metastatic tumors.

Previously, we successfully applied combinational chemotherapy with a P-gp inhibitor, HM30181A, and paclitaxel (a P-gp substrate) to the treatment of brain tumors including metastatic tumors (15), however there was a defined therapeutic window for treatment with P-gp inhibitor. Accordingly, we designed this study to confirm that P-gp on brain endothelial cells determines the chemosensitivity of brain metastatic tumors and to determine whether the therapeutic window is due to responses of the brain microenvironment to P-gp inhibitor treatments. In this study, we found that P-gp in the brain microenvironment and response of brain microenvironment to P-gp inhibitor are important factors determining therapeutic effect of P-gp inhibitor on brain metastatic tumors.

Materials and methods

Chemicals and cells. Tariquidar and HM30181A (CAS no. 849675-87-2) were provided by Hanmi Pharm. Co. (Seoul, Korea). The human breast cancer cell line, MCF7 [HTB-22, American Type Culture Collection (ATCC), Manassas, VA], its doxorubicin resistant sub-clone, MCF7/Dox, and the mouse brain endothelial cell line, bEnd.3 (CRL-2299, ATCC) were grown in 10% FBS/DMEM (Cambrex, East Rutherford, NJ). The mouse melanoma cell line, K1735 (Dr I.J. Fidler, M.D. Anderson Cancer Center) and the human brain microvascular cell line [HBMEC, ACBRI376, Applied Cell Biology Research Institute (ACBRI), Kirkland, WA] were cultured in 10% FBS/RPMI-1640 (Cambrex) and Complete Serum-Containing Medium Kits (ACBRI), respectively.

Rhodamine 123 (Rh123) efflux assay. The Rh123 efflux assay initiated at confluence. Cells were incubated in serum-supplemented growth medium containing 1 μM Rh123 (Invitrogen Co., Carlsbad, CA) and various concentrations of a P-gp inhibitor. After incubation, cells were washed with PBS and then incubated in serum-supplemented growth medium containing the same amount of the P-gp inhibitor. After 1 h, cells were lysed and the lysates were assayed at 485/535 nm.

In vitro cytotoxicity assay. After $1x10^4$ cells were cultured for 24 h, the media were replaced with paclitaxel-containing growth media with/without a P-gp inhibitor. Seventy-two hours later, the number of viable cells was determined using a Cell Counting Kit (Dojindo, Kumamoto, Japan).

MTT assay. Culture medium was replaced by the control salt solution containing 5 mg/ml MTT. After 2 h, the cells were dissolved and the absorbance was measured at 570 nm.

RT-PCR. Total RNA was extracted using the TRIzol reagent (Invitrogen Co.). First-strand cDNA was synthesized using the M-MLV reverse transcriptase (Promega, Madison, WI) and an Oligo-dT primer. One microliter of the first-strand cDNA reaction mixture and 0.2 μM of each primer set were used for PCR. Primers: i) P-gp (mouse): forward: 5'-AGGAA GACATGACCAGGTATGC-3', reverse: 5'-CCAACATCGT GCACATCAAAC-3'; ii) P-gp (human): forward: 5'-AGCAT CTGTGAACCACAT-3', reverse: 5'-GTTGCTGTTCTACC GCTGG-3'; iii) MRP1: forward: 5'-ACTCATTCAGCTCGT CTTGTCC-3', reverse: 5'-TCAACCCTGTGATCCACC AGA-3'; iv) GAPDH: forward: 5'-ACCACAGTCCATGCC ATCA-3', reverse: 5'-TCCACCACCCTGTTGCTGTA-3'. The PCRs were limited to 25 cycles.

Immunocytochemistry. Cells were grown on 1.2 mm cover glasses. MCF7, MCF7/Dox, bEND.3 or K1735 cells were fixed in 4% paraformaldehyde/PBS for 30 min and HBMECs were fixed in ice-cold methanol/acetone (1:1) for 10 min. After incubation in 0.2% Triton X-100/PBS for 15 min and then in 5% normal goat serum/PBS for 1 h, cells were treated by a mouse anti-P-gp monoclonal antibody (1:50, M3521, Dako, Glostrup, Denmark) diluted in 5% normal goat serum/PBS for 2 h at room temperature. An Alexa488-labeled goat anti-mouse IgG was used as secondary antibody (1:200, Invitrogen Co.).

Animals and intracerebral/subcutaneous implantation of tumor cells. Specific pathogen-free male C3H mice (6 weeks old) were used. Experiments were conducted in accordance with the 'National Institute of Health Guide for the Care and Use of Laboratory Animals' (NIH publication no. 80-23, revised in 1996). Intracerebral implantation of K1735 cells was performed as previously described (16). Briefly, anesthetized mice were secured in a rodent stereotactic frame and a hollow guide screw implanted into a small drill hole made at 2 mm left and 1 mm anterior to the bregma. Cells ($5x10^4$) in 10 μl HBSS were injected through this guide screw into the white matter at a depth of 2 mm. To establish the subcutaneous tissue tumor model, $1x10^6$ K1735 cells in 100 μl HBSS were implanted into the subcutaneous tissue.

Drug administration. One day after the inoculation of tumor cells, mice were randomized and received one of the following treatments: a) P-gp inhibitor vehicle (PEG200-HCl 15%) oral administration; b) 8 mg/kg paclitaxel intraperitoneal injection; c) 16 mg/kg HM30181A oral administration; or d) a combination of 16 mg/kg HM30181A oral administration and 8 mg/kg paclitaxel intraperitoneal injection. Mice were treated twice a week for 28 days.

Specimen harvesting and immunohistochemistry. Brains were harvested and processed for paraffin or frozen embedding. The tumor volume was recorded (largest width^2 x largest length x 0.5). The frozen blocks were cut into 8 μm coronal sections. Sections were thaw-mounted onto gelatin coated glass slides and fixed with acetone for 5 min. Immunohistochemistry was performed according to the methods used in the immunocytochemistry. The mouse anti-P-gp monoclonal antibody (M3521, Dako) and a rat anti-mouse CD31 monoclonal antibody (1:200, 557355, BD Pharmingen, Franklin Lakes, NJ) were used as primary antibodies and the Alexa488-labeled goat anti-mouse IgG and an Alexa594-labeled goat anti-rat IgG (1:200, Invitrogen Co.) were used as secondary antibodies. In some sections, P-gp was visualized according to the avidin-biotin complex (ABC) method, using an ABC kit (Vectastain™, Vector Laboratories, Berlingame, CA), and developed for peroxidase reactivity using 3,3'-diaminobenzidine (Sigma, St. Louis, MO).

Data analysis. To determine whether observed P-gp immunoreactivity changes were statistically significant, we randomly selected five tumor areas of each mouse, and determined mean staining densities of brain endothelial cells using the NIH image program (Scion Image). Mean staining density is the sum of the gray values of all the pixels in the selected area that was divided by the number of pixels within the area. Averages of mean staining densities of each animal were then calculated. All statistical analyses were performed using the Student's t-test. $P<0.05$ was considered statistically significant.

Results

Endothelial cells in K1735 brain tumor masses express P-gp. As shown in Fig. 1, the endothelial cells of the subcutaneous tissue did not express P-gp, but those of the brain expressed P-gp. When K1735 tumor cells were injected to these tissues

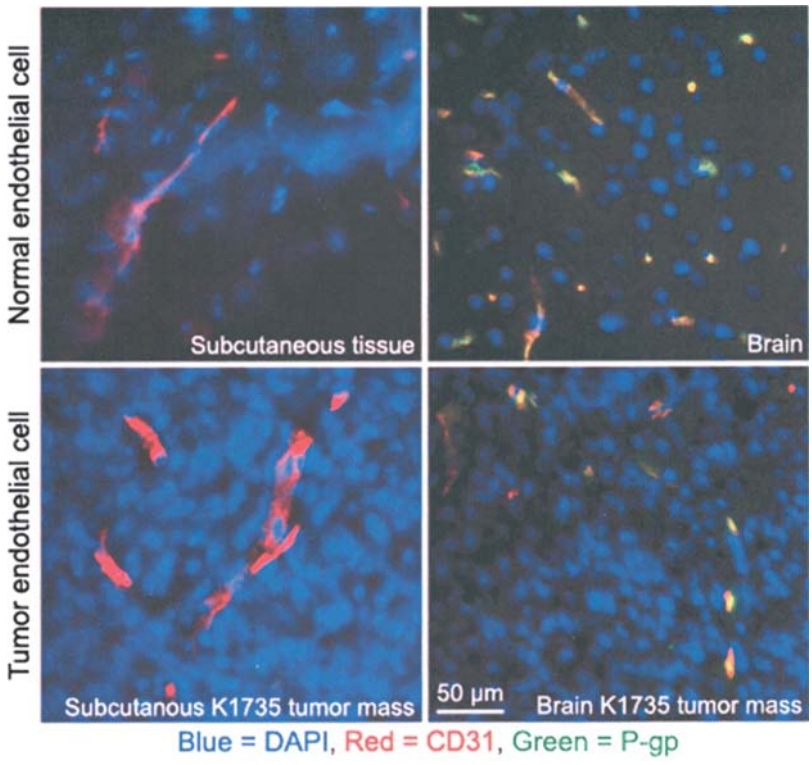

Figure 1. CD31 and P-gp expression in the subcutaneous tissue, brain gray matter (cerebral cortex), and K1735 tumor mass in subcutaneous tissue and brain. Tissues were stained with anti-CD31 (red), anti-P-gp (green) antibody, and DAPI (blue, nuclei).

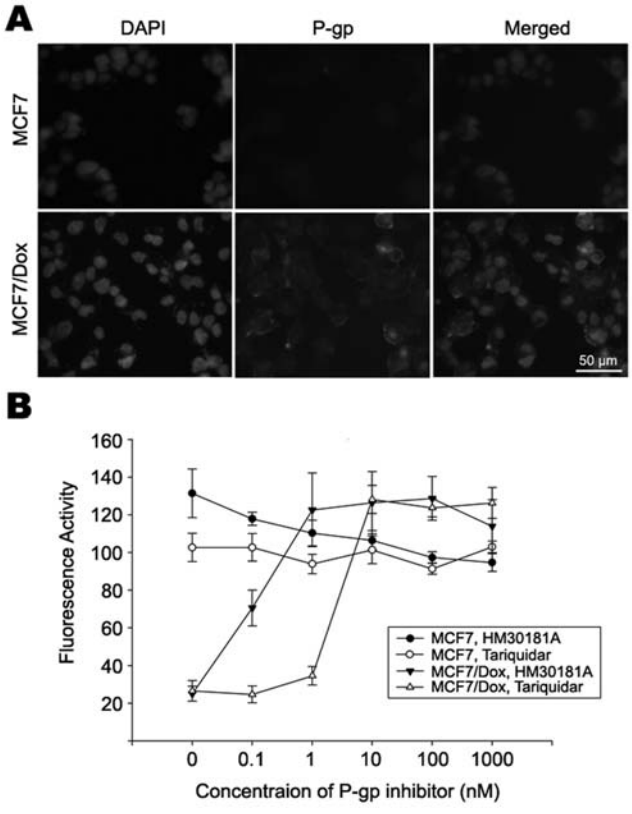

Figure 2. P-gp in MCF and MCF7/Dox and its activity. A, MCF7 and MCF7/Dox cells were stained with anti-P-gp antibody and DAPI (nuclei). B, MCF7 and MCF7/Dox cells were treated with 0.1, 1, 10, 100, or 1000 nM P-gp inhibitor (tariquidar or HM30181A) and 1 μM Rh123 (each group n=8) for 1 h. Rh123 retained in cells was quantified. Mean ± standard deviation.

and P-gp expressions of endothelial cells in K1735 tumor masses were examined, the P-gp expression patterns were not changed (Fig. 1). K1735 cells did not express P-gp in the subcutaneous tissue or the brain.

HM30181A activity. HM30181A is a newly developed competitive P-gp inhibitor (17,18). To confirm HM30181A's inhibitory activity, we performed Rh123 efflux assay with MCF7 and MCF7/Dox. MCF7/Dox is a P-gp expressing doxorubicin resistant sub-clone of MCF7 (Fig. 2A). In the Rh123 efflux assay (Fig. 2B), MCF7 cells did not show any significant Rh123 accumulation changes regardless of the type or concentration of P-gp inhibitor. However, MCF7/Dox cells showed Rh123 accumulations only in the presence of P-gp inhibitor. Rh123 retained in MCF7/Dox cells was increased more than 4 times at 10 nM tariquidar (a third-generation P-gp inhibitor) and 1 nM HM30181A compared with control ($P<0.0001$). In addition, 0.1 nM HM30181A increased Rh123 accumulation in MCF7/Dox cells about 2-fold ($P<0.0001$).

P-gp in bEnd.3 mouse brain endothelial cells and K1735 mouse melanoma cells. We examined the expression and function of P-gp in bEnd.3 and K1735 cells. P-gp and its mRNA were checked by immunocytochemistry and RT-PCR, respectively (Fig. 3A and B). P-gp and its mRNA were detected only in bEnd.3 cells. bEnd.3 cells showed dose-dependent increase in Rh123 accumulation in the presence of P-gp inhibitor (Fig. 3C). As HM30181A effectively inhibited P-gp function at lower concentration than tariquidar, we used HM30181A in our following experiments.

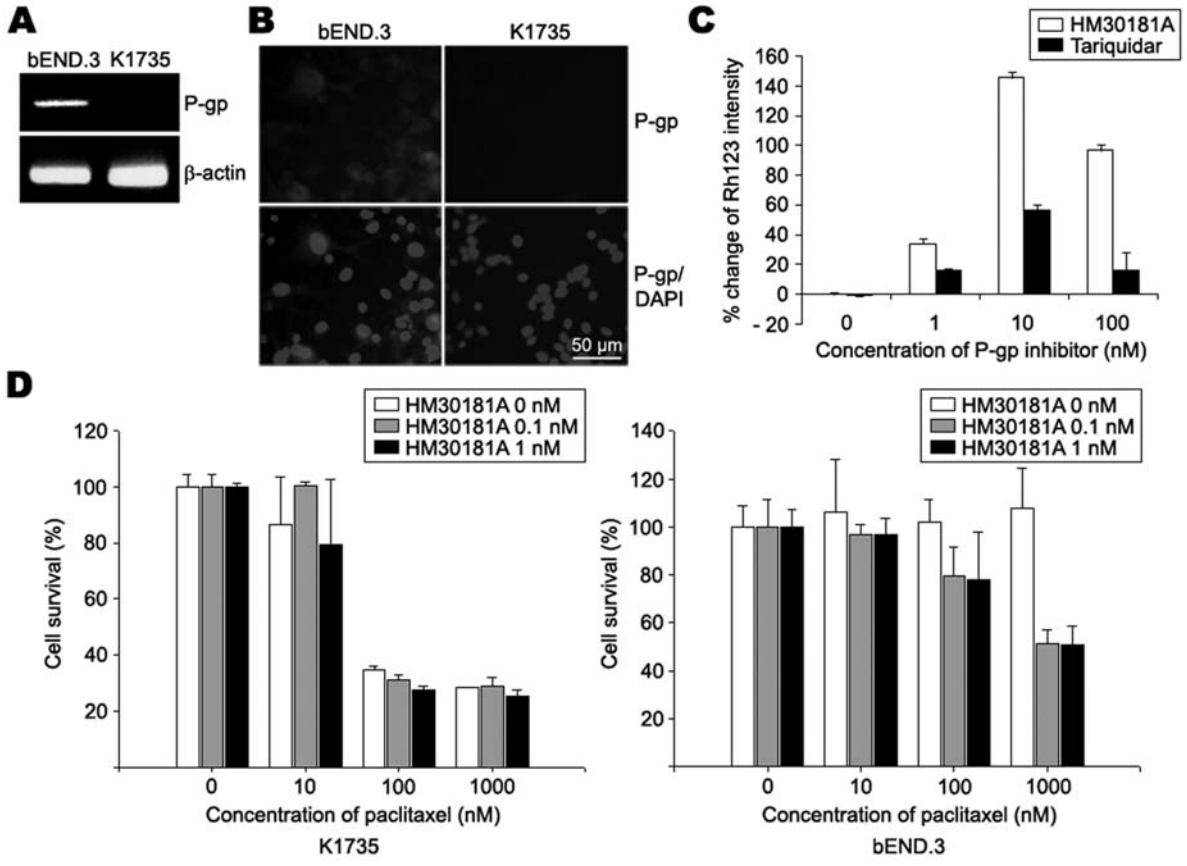

Figure 3. Effect of paclitaxel and HM30181A on the survivals of K1735 and bEnd.3. A, P-gp mRNA was detected by RT-PCR in bEnd.3 but not in K1735. ß-actin, internal control. B, bEnd.3 and K1735 cells were stained with anti-P-gp antibody and DAPI (nuclei). C, bEnd.3 cells were treated with 1, 10, or 100 nM P-gp inhibitor (tariquidar or HM30181A) and 1 μM Rh123 (each group n=4). % changes of Rh123 intensities {= [(Rh123 intensities - mean of NC Rh123 intensities)/mean of NC Rh123 intensities] x 100} were compared. D, K1735 and bEnd.3 cells were treated with 10, 100, or 1000 nM paclitaxel with/without 0.1 or 1 nM HM30181A (each group n=3). Cell survival was determined by CCK method 72 h after the drug treatments. Height, mean; error bar, standard deviation.

bEnd.3 cells are resistant to paclitaxel but K1735 cells are not, the resistance is overcome by HM30181A in vitro. To determine whether K1735 and bEnd.3 cells show paclitaxel resistance, we compared the survival of K1735 and bEND.3 cells in the various concentrations of paclitaxel and HM30181A (Fig. 3D). Compared with control (0 nM HM30181A), paclitaxel potently inhibited the survival of K1735 cells by about 65% at 100 nM and 72% at 1000 nM in a dose-dependent manner. HM30181A did not make any significant changes in the survival of K1735 cells. In contrast, bEnd.3 cells did not show any decreases in survival by the paclitaxel treatment without HM30181A. However, treatment of 0.1 or 1 nM HM30181A led to 20 and 42% inhibition of survival at the 100 nM and 1000 nM paclitaxel treatment, respectively.

K1735 brain metastasis is resistant to paclitaxel and P-gp inhibitor reverses the resistance in vivo. Paclitaxel sensitive K1735 cells were implanted into the subcutaneous tissue (primary tumor site of melanoma) or brain, treatments with paclitaxel (8 mg/kg intraperitoneal injection) and/or HM30181A (16 mg/kg oral administration) were performed (twice a week for 28 days), and volumes of tumor masses were measured one day after the last treatment. In the subcutaneous tumor model (Fig. 4B left) the average tumor volume of paclitaxel-treated mice was 31% of that of control mice ($P<0.01$). The tumor volume of HM30181A-treated mice was also decreased, but this was not statistically significant. The average tumor volume of paclitaxel/HM30181A co-treated animals was 81% smaller than that of controls ($P<0.01$), which was similar with that of the paclitaxel only-treated group. In the brain metastasis tumor model (Fig. 4A and B right), either paclitaxel or HM30181A alone had no effect. In contrast, the paclitaxel/HM30181A combined therapy dramatically decreased tumor growth by 49% ($P<0.05$). During treatment, no significant systemic side-effects were observed.

Effects of P-gp inhibitor treatment on P-gp expression and function of brain endothelial cells. To observe responses of endothelial cells of K1735 brain tumor masses to P-gp inhibitor treatment, we measured the changes in P-gp expression and function of the HBMECs in response to HM30181A treatment *in vitro*. Tumor cells use the normal brain vasculature at the early stages of metastasis (14). Therefore, the response of the HBMECs would mimic the response of brain tumor endothelial cells. The P-gp mRNA levels were determined by RT-PCR after a 3- or 48-h exposure to 0.1 or 10 nM HM30181A (Fig. 5A). The MRP1 (19) was examined in parallel. HM30181A treatment did not affect the MRP1 and

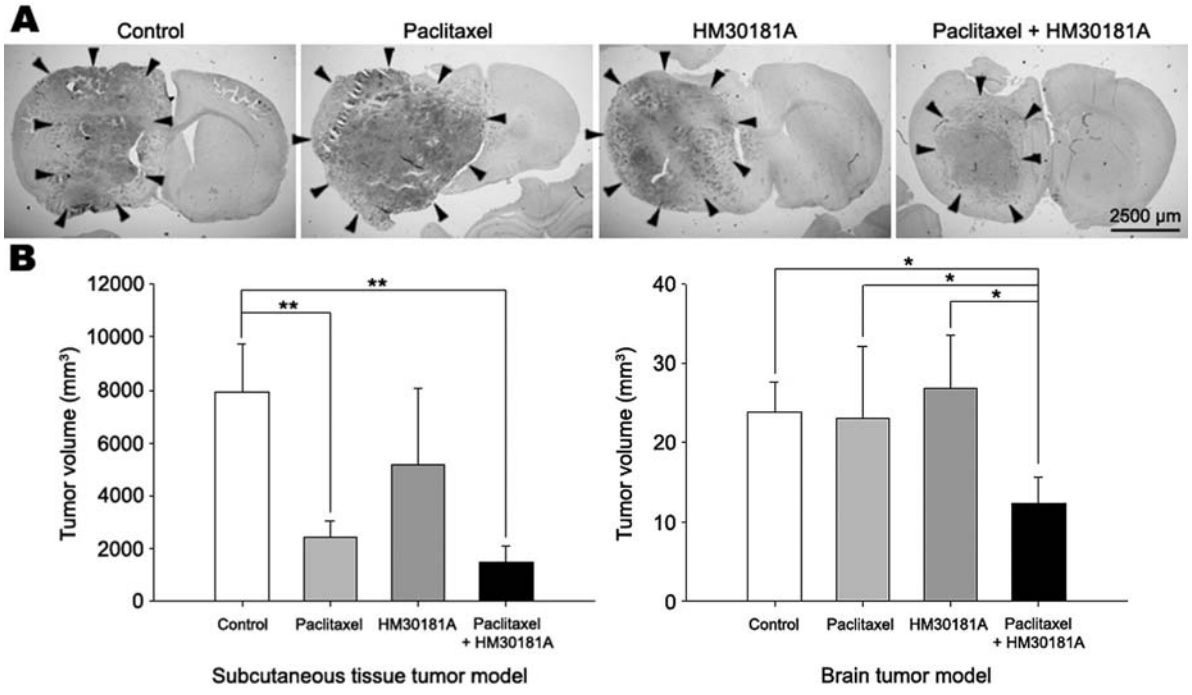

Figure 4. The effect of paclitaxel and HM30181A combined therapy on tumor growth in subcutaneous tissue and brain metastasis tumor model. A and B (right), K1735 cells were implanted into the brain parenchyma and one day after the tumor cell injections, the mice received HM30181A vehicle (control, n=8), 8 mg/kg paclitaxel intraperitoneal injection (n=8), 16 mg/kg HM30181A oral administration (n=8), or 16 mg/kg HM30181A oral administration and 8 mg/kg paclitaxel intraperitoneal injection combined (n=9) therapy. The drugs were administrated two times per week and tumor volume was measured at 28 days after the tumor injections. $^{*}P<0.05$. B (left), K1735 cells were implanted into the subcutaneous tissue, the same treatments with the brain tissue tumor model were performed (control, n=8; paclitaxel, n=8; HM30181A, n=7; paclitaxel/HM30181A, n=8), and tumor volumes were measured. $^{**}P<0.01$. Height, mean; error bar, standard deviation.

GAPDH mRNA levels (Fig. 5A). However, a 3-h exposure to 0.1 or 10 nM HM30181A significantly reduced the P-gp mRNA level (each group n=3, $P<0.05$). The effect of the 0.1 nM HM30181A treatment persisted until the 48 h time point. In contrast, the P-gp mRNA level re-adjusted to a normal level (similar to control) when exposed to 10 nM HM30181A for 48 h.

When the P-gp levels were assessed by immunocytochemistry, the P-gp protein levels closely followed the mRNA levels (Fig. 5B). We also measured P-gp activity in the HBMECs using Rh123 efflux assay. Although P-gp activity was significantly inhibited by the HM30181A treatments in the other groups ($P<0.05$), the capacity for transporting Rh123 was re-adjusted to a normal level (similar to control) when exposed to 10 nM HM30181A for 48 h (Fig. 5C left). The P-gp function changes were not due to cytotoxicity of HM30181A (Fig. 5C right).

In vivo responses of the brain microenvironment to P-gp inhibitor treatment. We checked whether P-gp expression of brain endothelial cells is also changed *in vivo* in response to HM30181A treatment and if the expression changes are related with the therapeutic results using the samples which we have obtained in our previous study (15). In the study, 32 mg/kg HM30181A + 16 mg/kg paclitaxel oral co-administration showed significant therapeutic effects on K1735 brain masses, while both 16 mg/kg HM30181A + 8 mg/kg paclitaxel and 48 mg/kg HM30181A + 32 mg/kg paclitaxel oral co-administration did not. As shown in Fig. 6, P-gp expression of brain endothelial cells in the K1735 brain masses was decreased in the 32 mg/kg HM30181A + 16 mg/kg paclitaxel group ($P<0.05$), however its level was re-adjusted to a normal level (similar to control) in the 48 mg/kg HM30181A + 16 mg/kg paclitaxel group. These results show that exposure to higher dose of HM30181A than the therapeutic window of HM30181A (15) induces P-gp expression on endothelial cells of K1735 brain tumor mass and this abolishes the therapeutic effect of HM30181A and paclitaxel combination treatment *in vivo*.

Discussion

In this study, we showed that P-gp on brain endothelial cells and BBB can confer multidrug resistance to tumors in the brain parenchyma even though these tumors, themselves, do not express P-gp (Figs. 1, 3, and 4). To reverse this conferred resistance we tested the combined therapeutic effect of paclitaxel and a competitive P-gp inhibitor, HM30181A using orthotopic *in vivo* models and found that the resistance can be inhibited by HM30181A (Fig. 4). These results demonstrate that P-gp on brain endothelial cells can be a good chemotherapeutic target to reverse the chemoresistance of brain metastatic tumors. In a previous study, we treated with HM30181A to increase the penetration of paclitaxel across the endothelial cells of brain tumor (15). However, the therapeutic results were disappointing with both lower (16 mg/kg) and higher (48 mg/kg) doses of HM30181A than the therapeutic window (32 mg/kg). The present data show that high-dose

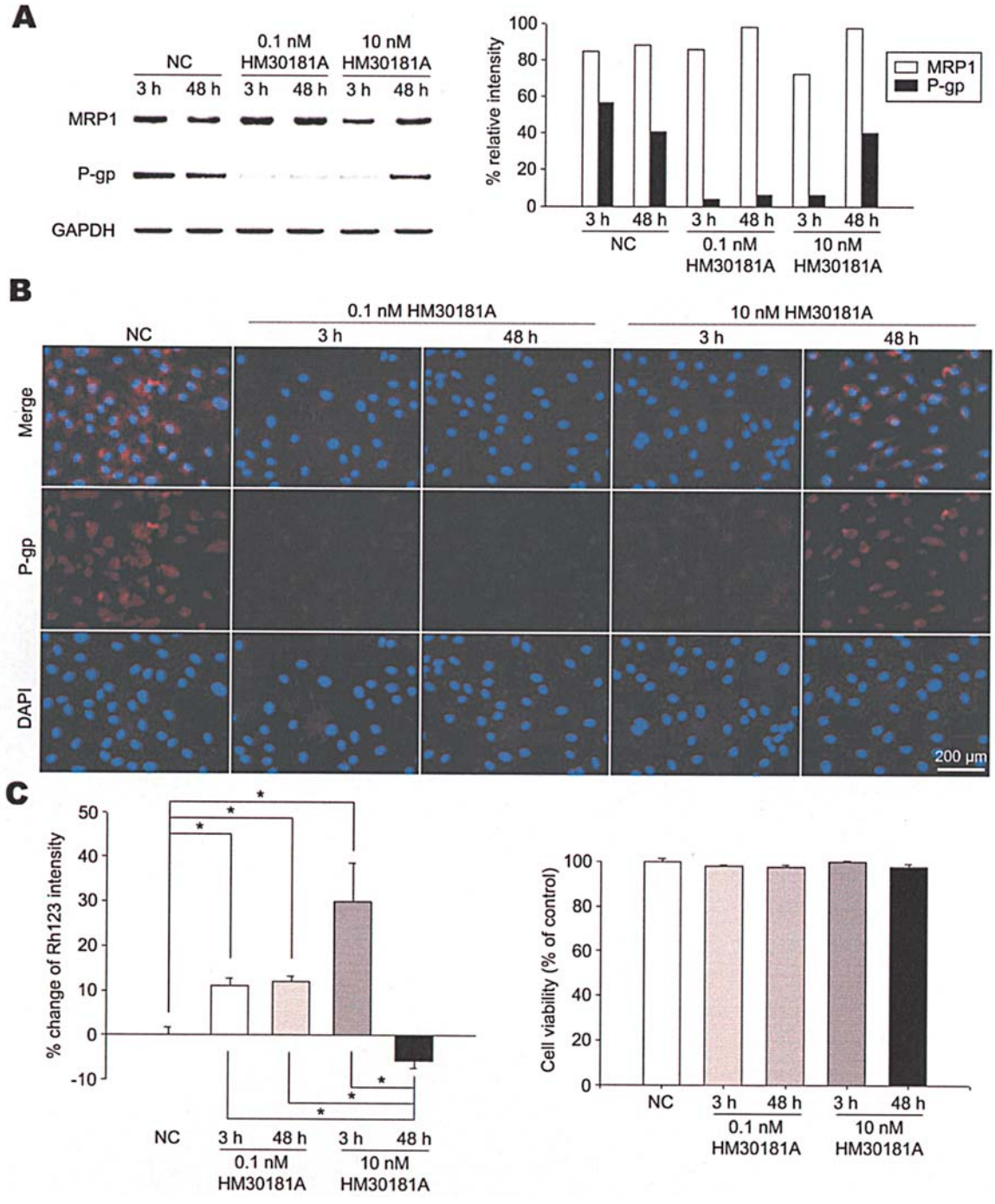

Figure 5. Effects of HM30181A on P-gp expression and function in brain endothelial cells. Growth medium was used as a negative control (NC). A, The P-gp, MRP1, and GAPDH mRNA levels were determined in HBMECs using RT-PCR. The signal intensities of the P-gp and MRP1 PCR products were divided by those of the GAPDH PCR products (% relative intensity) and compared. B, The P-gp levels in HBMECs were assessed by immunocytochemistry. P-gp, red; DAPI (nuclei), blue. A and B, one of three experiments is shown (all showed similar results). C (left), Rh123 efflux assay was performed in HBMECs. % changes of Rh123 intensities {= [(Rh123 intensities - mean of NC Rh123 intensities)/mean of NC Rh123 intensities] x 100} were compared. C (right), the viability of HMBECs was checked using MTT assay. C, Each group n=3. Height, mean; error bar, standard deviation. *P<0.05.

HM30181A treatment results in paradoxical P-gp expression in brain endothelial cells *in vitro* and *in vivo* (Figs. 5 and 6). This would prevent paclitaxel from penetrating into brain tumors and could explain our previous data. Therefore, induction of P-gp expression on brain endothelial cells in response to high dose P-gp inhibitor treatment can be a factor that makes influences on the therapeutic effects of P-gp inhibitor.

P-gp is the best characterized molecule responsible for multidrug resistance of tumors. However, it is also expressed by endothelial cells in the brain and in the tumor associated endothelial cells (TAECs) in brain tumors (20,21). Unlike the BBB at normal brain, brain tumor vasculature has lost its conformation to show wider diffusion of contrast agents and investigated proteins compared with healthy tissues. But, some chemotherapeutic agents still hardly penetrate into the

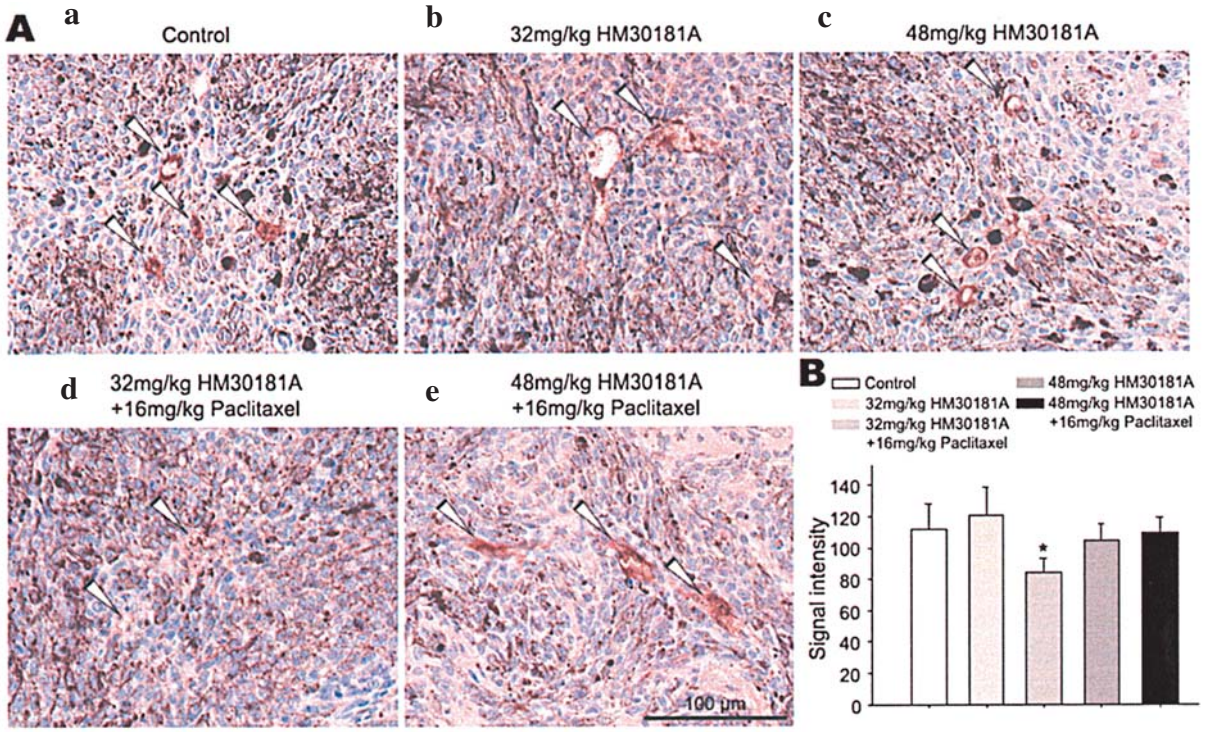

Figure 6. Effects of P-gp inhibitor on P-gp expression of endothelial cells in K1735 brain tumor masses. A, K1735 cells were implanted into the brains of C3H mice and one day after the tumor cell injections, the mice received oral administration of (a) P-gp inhibitor vehicle (control, n=5); (b) 32 mg/kg paclitaxel (n=4); (c) 48 mg/kg HM30181A (n=4); (d) 32 mg/kg HM30181A + 16 mg/kg paclitaxel (n=5); or (e) 48 mg/kg HM30181A + 16 mg/kg paclitaxel (n=5). The drugs were administrated two times per week for 28 days. P-gp expression of endothelial cells in K1735 brain masses were analyzed by immunohistochemistry (arrowheads). B, P-gp staining densities of brain endothelial cells were analyzed. Height, mean; error bar, standard deviation. *P<0.05.

brain tumor matrix (20). In our study, we demonstrated that the P-gp expression of brain TAECs could have major role in the chemoresistances of brain tumors.

There are some reports that P-gp expression in TAECs are different between primary and metastatic brain tumors: the TAECs of gliomas express high levels of P-gp, in contrast, those of brain metastatic tumors hardly express P-gp (22,23). In one report, P-gp was immunodetected in 60 human primary tumors at the same levels as normal brain tissue, while P-gp levels were 70 and 95% lower in brain metastases from melanomas and lung adenocarcinomas, respectively (24). In spite of those reports, the brain microenvironment is supposed to have crucial role in chemoresistance of tiny metastatic lesion. At the first stage of brain metastasis, tumor cells use the normal brain vasculature, so, comparable level of P-gp is expressed and BBB is intact (14,25). For this reason, brain metastasis is often discovered after complete remission of primary cancer. Furthermore P-gp expressions by the TAECs of brain metastatic tumors were detected in some cases (21). Thus, our brain metastasis model could translate the condition that metastatic cancer cells use the brain endothelial cells and this model could be helpful in the treatment methods development for patients who have or are supposed to have tiny brain metastases.

Many P-gp blocking agents have been applied to increase the efficacy of cytotoxic agents. However, the results have been disappointing because some agents have low binding affinities, others compete with the cytotoxic agents for cytochrome P450 3A4 (19). A third generation of P-gp inhibitors is in clinical development and includes tariquidar (26), zosuquidar (27), laniquidar (28), valspodar (29), and ONT-093 (30). These share a high potency and specificity for the P-gp, however, tariquidar (31) and valspodar (29) had failed to restore sensitivity of chemoresistant tumors to anthracycline or taxane in recent trials. It is possible that additional factors influence their effects *in vivo*. Alternatively, the increased P-gp expression induced by a P-gp inhibitor could explain the disappointing results.

In this report, we confirmed that P-gp in brain microenvironment could transform chemosensitive tumors in other tissue into chemoresistant tumors in the brain parenchyma using *in vivo* models. We have also demonstrated that P-gp expression in brain endothelial cells can be increased by high-dose P-gp inhibitor treatment, which was related with the therapeutic effect loss of high-dose P-gp inhibitor treatment. These results suggest that P-gp in the brain microenvironment has crucial roles in the brain metastatic tumor chemoresistance and brain microenvironment responses to P-gp inhibitor treatment should be considered in the development of brain endothelial cell-targeted chemotherapy using P-gp inhibitor.

Acknowledgements

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