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Anticancer activity of PEGylated matrix metalloproteinase cleavable peptide-conjugated adriamycin against malignant glioma cells

Seung-Ho Lim[a], Young-Il Jeong[b], Kyung-Sub Moon[a], Hyang-Hwa Ryu[b], Yong-Hao Jin[b], Shu-Guang Jin[b], Tae-Young Jung[a], In-Young Kim[a], Sam-Suk Kang[a], Shin Jung[a,b,c,*]

[a] Department of Neurosurgery, Chonnam National University Hwasun Hospital & Medical School, Republic of Korea
[b] Brain Tumor Research Laboratory, Chonnam National University Hwasun Hospital & Medical School, Republic of Korea
[c] Chonnam National University Research Institute of Medical Sciences, Chonnam National University Hwasun Hospital & Medical School, Republic of Korea

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ABSTRACT

Although matrix metalloproteinases (MMPs) play a crucial role in the invasion and growth of malignant gliomas, their increased activity in tumor environment can be used as a specific target for chemotherapy. We investigated whether polymer–drug conjugates formed via MMP-cleavable peptide linkages could provide MMP-responsive tumor targeting and cytotoxicity for malignant glioma cells. One end of an MMP-cleavable peptide was attached to the end of methoxy polyethylene glycol (MPEG) while the other end was attached to adriamycin (ADR). The release of drugs in the presence of conditioned media of U87MG cells was investigated. The cytotoxicities of the MMP-cleavable MPEG–peptide–ADR (PPA) conjugates and non-cleavable MPEG–ADR (PA) conjugates were investigated using U87MG cells. The ^{1}H nuclear magnetic resonance (NMR) spectra confirmed the conjugation of the two ends of the peptide to the ends of MPEG and ADR, respectively. Gelatin zymography showed that MMP-2 was strongly expressed in the media of U87MG cells. The PA conjugate did not release ADR either in the phosphate buffered saline (PBS) or conditioned media of U87MG cells. The PPA conjugate released ADR in the presence of the conditioned media of U87MG cells, but not in PBS only. In the cytotoxicity test using U87MG cells, ADR and PPA conjugate showed similar anti-proliferative activities, while the cytotoxicity of PA conjugate was lower than that of ADR. Considering that the cytotoxicity of the PPA conjugate was similar to that of ADR, MMP-cleavable polymer–drug conjugates can be used as targeting carriers for the purpose of inhibiting the proliferation of malignant glioma cells.

1. Introduction

The infiltrative nature of gliomas is responsible for the high morbidity and mortality associated with these tumors. Surgical debulking of the tumor constitutes only a temporizing measure, as microscopic infiltrated foci of the tumor will lead to eventual recurrence, often in areas that are surgically inaccessible. Additionally, traditional adjuvant treatments such as postoperative radiotherapy or chemotheraphy using temozolomide can lead to the increase of invasion-related peptide expression, angiogenesis-related and multidrug resistance-related gene expression. They may promote invasion and malignant transformation of tumor cells (Bernstein and Woodard, 1995; Trog et al., 2005, 2006a,b; Wild-Bode et al., 2001; Woodhouse et al., 1997). As the result, patients afflicted with high grade gliomas are faced with a poor prognosis, with less than 10% of them surviving beyond 2 years.

Stopping tumor cells from invading and metastasizing through chemoprevention is a new goal of cancer treatment. Various novel strategies have been studied over the past two decades to treat gliomas, such as sustained release implant systems, microspheres, nanoparticles, liposomes, and polymer–drug conjugates (Brem et al., 1991; Chau et al., 2004; Emerich et al., 2000; Kreuter, 1993; Zhou and Huang, 1992). Among them, polymer–drug conjugates have remarked themselves in the targeted delivery of anticancer drugs to the tumor site (Chau et al., 2004; Duncan et al., 1992).

The aim of this study was to synthesize a PEGylated peptide–ADR conjugate and to test it against various glioma cell lines. Since MMP-2 and/or -9 are known to play a crucial role in the invasion of brain tumors (Deryugina et al., 1997; Lampert et al., 1998; Nakano et al., 1995), MMP-2 cleavable peptides (Pro-Val-Gly-Leu-Ile-Gly), well characterized by the Langer group (Chau et al., 2004), were conjugated to the end of MPEG and then ADR was conjugated to the end of this peptide. We expected that ADR would be released from the PPA conjugates only in the presence of

* Corresponding author at: Department of Neurosurgery, Chonnam National University Hwasun Hospital, 160, Ilsim-ri, Hwasun-eup, Hwasun-gun, Jeollanam-do 519-809, Republic of Korea. Tel.: +82 61 379 7666; fax: +82 61 379 7673.
E-mail address: sjung@chonnam.ac.kr (S. Jung).

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MMP-2 and/or -9. Strategies involving the use of MMP-responsive drug release may provide the possibility of tumor targeting correlated with a specific enzyme and inhibit the invasion of malignant glioma cells.

2. Materials and methods

2.1. Materials

Methoxy poly(ethylene glycol) N-hydroxysuccinimide (MPEG-NHS, MW = 5000 g/mol) was purchased from Sunbio Chem. Co., Korea. Adriamycin (ADR), dimethylsulfoxide (DMSO), DMSO-d form, dialysis tubes (MW cut-off sizes: 2000 g/mol, 12,000 g/mol), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC) were purchased from Sigma Chem Co., USA. Dulbecco's modified-Minimum Eagle's medium (DMEM) was purchased from GIBCO (Invitrogen Co., USA). MMP-cleavable peptide (peptide sequence: Pro-Val-Gly-Leu-Ile-Gly) was purchased from Peptron Co., Korea. All other chemicals and reagents used in the experiments were of extra reagent grade and were purchased from Sigma Co. Ltd., USA.

2.2. Synthesis of MPEG–peptide–adriamycin (PPA)

The conjugation of the peptide to MPEG-NHS was performed as follows: the peptide and MPEG-NHS (1.2:1, molar ratio) were mixed in 0.5 M carbonate buffer (pH 8.5) and stirred at 4 °C overnight. After that, the solution was dialyzed for 2 days at 4 °C and lyophilized. The resulting powder and ADR (1: 1.2 molar ratio) were mixed in dry DMSO with EDAC and stirred overnight (ADR was pretreated with a trace amount of TEA to remove any HCl). After the reaction, the reaction solution was dialyzed against 3 L of acetate buffer (pH 5.5, 0.1 M) for 3 h and then against distilled water for 2 days. Then, the MPEG–peptide–ADR (PPA) conjugates were lyophilized for 3 days and stored in a refrigerator until use. The attachment of ADR to the end of the peptide was confirmed by UV spectroscopy (489 nm, UV-1201 spectrophotometer, Shimadzu Co., Japan). The conjugation yield was about 95.0%, i.e. 95.0% of the MPEG–peptide was attached to the ADR. Conjugation yield (%, w/w) = [(residual mol% of ADR/mol% of MPEG–peptide)] × 100.

2.3. Synthesis of MPEG–ADR

For the purpose of comparison, an MMP-non-cleavable conjugate of MPEG–ADR was synthesized. To accomplish this, MPEG-NHS and ADR were dissolved in dry DMSO (molar ratio = 1:1.2) and reacted overnight at room temperature. Before the reaction, TEA was added to the ADR/DMSO solution. After the reaction, the reaction solution was dialyzed against 3 L of acetate buffer (pH 5.5, 0.1 M) for 3 h and then against distilled water for 2 days. After that, the MPEG–ADR (PA) conjugates were lyophilized for 3 days and then stored in a refrigerator until use. The conjugation yield was about 97%. All of the procedures were performed under dark conditions.

2.4. ^{1}H nuclear magnetic resonance (NMR) spectra measurement

The ^{1}H NMR spectra were recorded with DMSO-d form (400 MHz NMR, Varian), in order to confirm the synthesis of PPA (MPEG–peptide–ADR) and PA (MPEG–ADR).

2.5. Cells

The malignant glioma cell lines, U251MG, U87MG, 9L, and C6, were obtained from the American Type Culture Collection (ATCC, USA). The cells were cultured and maintained under 5% CO_2 at 37 °C.

2.6. Gelatin zymography

The various cell lines were seeded onto 10 cm tissue culture plates and cultured under 5% CO_2 at 37 °C in the presence of serum. The cells were grown and enriched on a culture plate (about 70% of the plate). Subsequently, the medium was replaced by 2 ml of serum-free DMEM per plate. After 48 h of incubation, the conditioned media was collected and ultracentrifuged to remove the cell debris. After this procedure, the cells were harvested to study the intracellular MMP expression, as described below. The centrifuged medium was used for the gelatin zymography assay, as follows: 50 μg of the total protein from the dialyzed solution was mixed with the sample buffer (50 mM Tris–HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol). It was electrophoresed on 8% denaturing sodium dodecyl sulfate (SDS) polyacrylamide gels containing 2 mg/ml of gelatin (type A, sigma). The gel was washed three times for 30 min (each?) in 2.5% Triton X-100: the gel was then incubated for 20 h at 37 °C in 50 mM of a buffer solution containing Tris–HCl (pH 7.5), 10 mM $CaCl_2$, and 200 mM NaCl. The gel was stained with Coomassie Brilliant Blue R-250 (0.2% Coomassie Brilliant Blue R-250, 20% methanol, 10% acetic acid in H_2O) and then destained (20% methanol, 10% acetic acid in H_2O).

For the preparation of the intracellular MMPs, the cells were harvested by treating them with Trypsin–EDTA and then centrifuged. The pellet was taken and homogenized in buffer solution (50 mM Tris–HCl (pH 7.5), 10 mM $CaCl_2$, 200 mM NaCl) using a homogenizer. 50 μg of the total protein from the homogenate supernatants was mixed with sample buffer (50 mM Tris–HCl, 2% SDS, 0.1% Bromophenol blue, 10% glycerol). It was electrophoresed on 8% denaturing sodium dodecyl sulfate (SDS) polyacrylamide gels containing 2 mg/ml of gelatin (type A, Sigma). The gel was washed three times for 30 min (each?) in 2.5% Triton X-100 and then incubated for 20 h at 37 °C in buffer solution containing 50 mM Tris–HCl (pH 7.5), 10 mM $CaCl_2$, and 200 mM NaCl. The gel was stained with Coomassie Brilliant Blue R-250 (0.2% Coomassie Brilliant Blue R-250, 20% methanol, 10% acetic acid in H_2O) and then destained (20% methanol, 10% acetic acid in H_2O).

2.7. Cell proliferation assay

The effect of free ADR, PA and PPA on cell growth was determined using an MTT cell proliferation assay. ADR was dissolved in 100% DMSO and diluted 100 times using DMEM. PA and PPA were dissolved in serum-free DMEM (at an equivalent concentration to ADR) and diluted to adjust the equivalent concentration of the free ADR. The brain tumor cell lines were seeded at a density of 1×10^4 per well in 96-well plates with serum-free DMEM and incubated overnight in a CO_2 incubator (5% CO_2 at 37 °C). After that, fresh medium containing each chemical was added. After 1 day of incubation, the cell proliferation assay, MTT Cell Titer 96, was performed. The absorbance was measured at 560 nm using a microtiter plate reader (Thermomax microplate reader, Molecular Devices).

2.8. Drug release study

For the drug release study in the presence or absence of the MMPs, U87MG cells or U251MG cells were grown and enriched on a culture plate. Subsequently, the medium was replaced by 2 ml of serum-free DMEM per plate. After 48 h of incubation, the conditioned media was collected and ultracentrifuged to remove the cell debris. The supernatants were dialyzed against phosphate buffer (pH 7.4, 0.1 M, MW cut-off size of dialysis tube: 12,000 g/mol) for 1 day to remove the phenol red (which is contained in the DMEM)

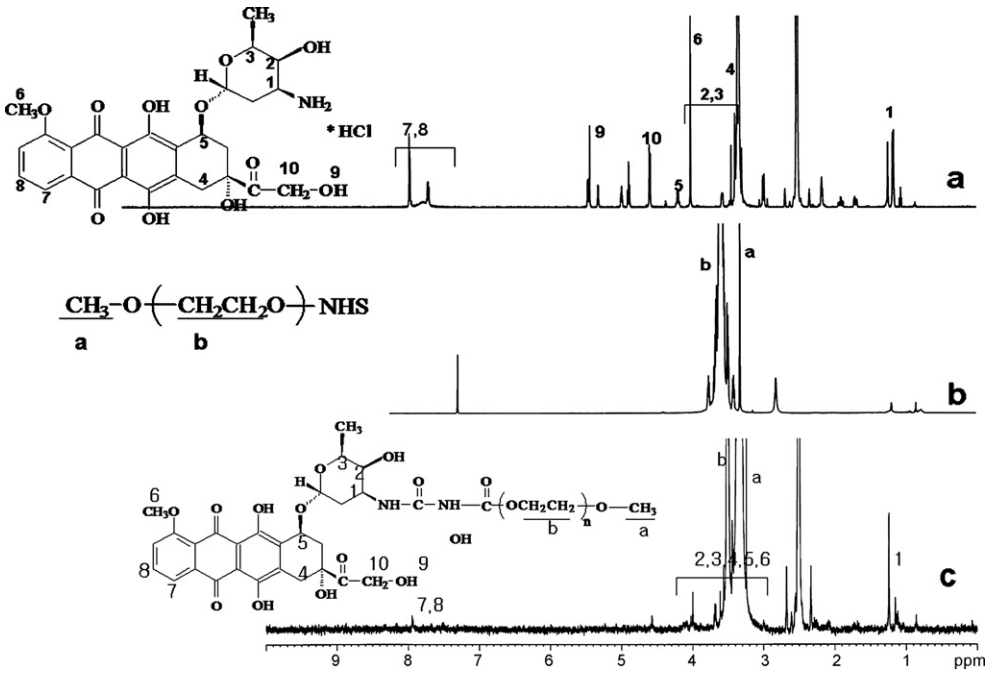

Fig. 1. ^{1}H NMR spectra of DOX (a), MPEG (b), and PA conjugates (c). All of the chemicals were dissolved in DMSO-d.

from the conditioned medium. To the dialyzed solution, ADR, PA, and PPA were added and dissolved at an equivalent concentration of ADR (5 mg ADR/5 ml of conditioned media) and this solution was introduced into the dialysis tube (MW cut-off size: 2000 g/mol). This dialysis tube was introduced into a 200 ml bottle containing 95 ml of PBS (pH 7.4, 0.1 M) and stirred at 100 rpm at 37 °C. The drug released to the outer phase of the dialysis tube was checked with a UV-spectrophotometer at 489 nm (UV-1201 Spectrophotometer, Shimadzu Co., Japan).

3. Results

3.1. Synthesis of PA and PPA conjugates

ADR was directly conjugated to the end of MPEG-NHS for the purpose of comparison (PA), as shown in Fig. 1. In the NMR results, ADR (Fig. 1(a)) has specific peaks at around 1.0–6.0 ppm, while MPEG (Fig. 1(b)) has specific peaks at around 3.7 ppm. As shown in Fig. 1(c), PA showed the specific peaks of both ADR and MPEG at around 1.0–5.0. This result showed that ADR was successfully conjugated to the end of MPEG. Fig. 2 shows the procedure used for the synthesis of PPA. The synthesis of PPA was composed of two steps. First, the MMP-cleavable peptide (Pro-Val-Gly-Leu-Ile-Gly) was conjugated to the active end of MPEG-NHS. Second, ADR was conjugated to the end of the peptide (PPA). MMP-sensitive peptide (Fig. 2(a)) has specific peaks at around 1–9 ppm. Fig. 2(b) showed both of peptide and ADR peaks at 3.7 ppm and around 1–5 ppm. As shown in Fig. 3(c), PPA has the peaks of MPEG, the peptide, and ADR. These results showed that the MMP-cleavable peptide was successfully conjugated between MPEG and adriamycin.

3.2. Gelatin zymography

The gelatin zymography results of the various glioma cell lines are shown in Fig. 3. As shown in Fig. 3, the conditioned media from the U87MG cells showed the strongest expression of MMP-2, since the molecular weight of MMP-2 is about 72,000. The conditioned media of the U251MG, 9L, and C6 cell lines revealed very weak band intensities for MMP-2. The cell lysates showed relatively weak intensities for all of the samples. Since the media of the U87MG cell culture showed the strongest MMP-activities, the conditioned media from the U87MG cells was used in the following experiments.

3.3. Drug release study

To study whether or not the conditioned media from the cell culture can affect the release of ADR by means of MMP-responsive degradation and liberation, the release of ADR from the PA and PPA conjugates in the presence or absence of the conditioned media of U87MG cells (Fig. 4(a)) or U251MG cells (Fig. 4(b)) was tested and the results are shown in Fig. 4. As shown in Fig. 4(a), no drug release of either PA or PPA was observed in the absence of the conditioned media of U87MG cells. In the presence of the conditioned media of U87MG cells (Fig. 4(a)), the release of ADR was observed from the PPA conjugates, while ADR was not released from the PA conjugates. These results indicate that the PPA conjugates exhibit MMP-responsive drug release behavior, while PA does not show MMP-responsive behavior. Fig. 4(b) shows the release of ADR from the PA and PPA conjugates in the presence of the conditioned media of U251MG cells. Since the U251MG cells showed negligible activities in MMP-2 (Fig. 3), the release of ADR from PPA was significantly lower than that observed in the case of the U87MG cells.

3.4. Cell cytotoxicity

In the cytotoxicity test using U87MG cells (Fig. 5(a)), the ADR and MMP-cleavable polymer–drug conjugates (PPA) showed similar cytotoxicities, while the MMP-non-cleavable PA was less cytotoxic to the U87MG cells than ADR. These results indicated that ADR release from PPA can responded to the expression of MMP and that the released ADR has similar cytotoxicity with free ADR. As shown in Fig. 5(b), both PA and PPA were less cytotoxic to U251MG cells than ADR. When the conditioned media of U87MG cells was added, however, PPA showed similar cytotoxicity to ADR.

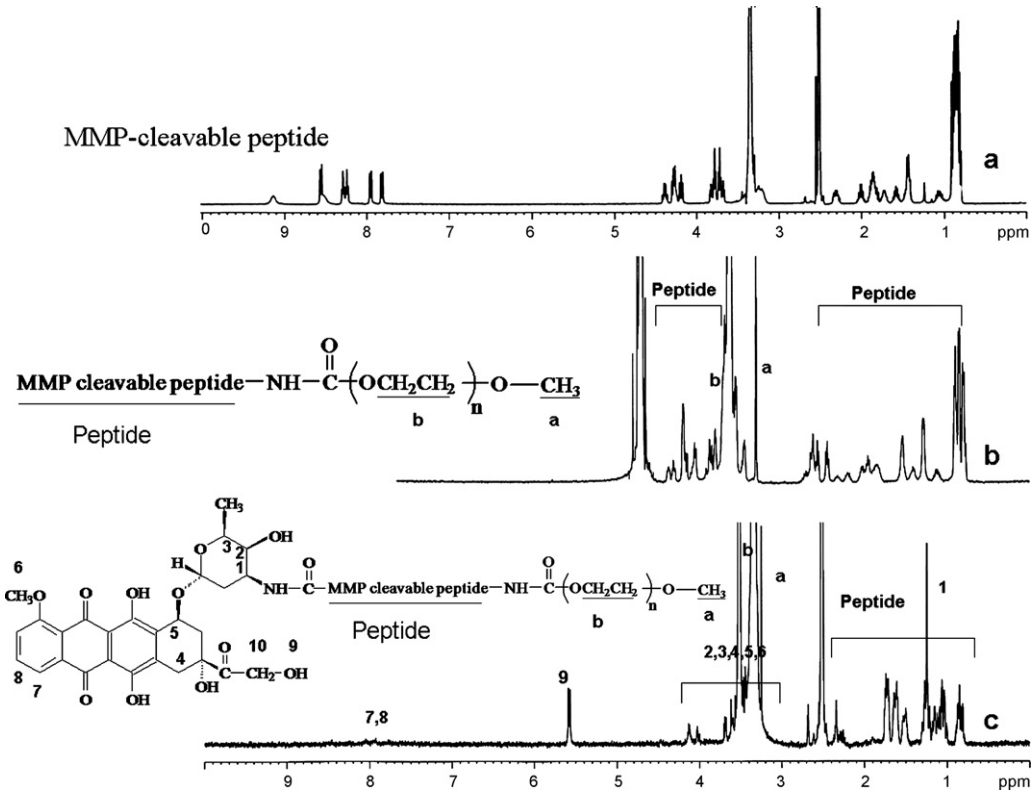

Fig. 2. [1]H NMR spectra of MMP-cleavable peptide (a), MPEG–peptide conjugate (b) and PPA conjugate (c). All of the chemicals were dissolved in DMSO-d. Sequence of MMP-cleavable peptide: Pro-Val-Gly-Leu-Ile-Gly.

4. Discussion

The chemotherapy of cancer is not always successful, the major reason for which is the metastasis of cancer cells to other organs and local invasion. Stopping tumor cells from invading and metastasizing through chemoprevention is a new goal of cancer treatment. In brain tumors, local invasion is the major factor involved in tumor growth and is more widespread than metastasis.

Based on the fact that it is rare for glioma cells to enter the subarachnoid space and the cerebral microvasculature, or metastasize to extracranial organs, local invasion is the major factor influencing glioma growth (Bernstein and Woodard, 1995; Woodhouse et al., 1997). The infiltrated cells also often escape surgical resection to lead to inevitable recurrence of glioma. Interestingly, the pathological grade of a glioma does not seem to be strictly correlated with the degree of local invasion, i.e. low-grade astrocytomas may show extensive infiltration of the adjacent brain tissue. In contrast, the tumor grade positively correlates with the proliferation rate. Proliferative and invasive behaviors are not determined by the same sequence of genetic events. Proliferation and cell motility may be interrelated but dichotomous behaviors in vitro. A motile phenotype in gliomas may occur at the expense of the proliferative activity of the cell. Thus, relapses and recurrences of brain tumors occur not only at the primary site, but also in distant locations.

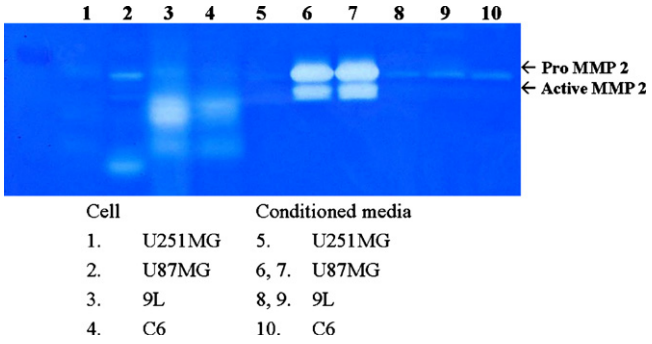

Fig. 3. Gelatin zymography of glioma cell lines. The white bands in the backgrounds indicate the presence of gelatinases (MMP-2). Tumor cells were confluently cultured in 10 cm plates with serum-free DMEM and gelatin zymography was performed with the media. Active MMP-2 was strongly expressed by U87MG.

A three step process of tumor cell invasion has been proposed: (1) receptor-mediated matrix adhesion, (2) degradation of the matrix by tumor-secreted hydrolytic enzymes (proteases), and (3) tumor cell locomotion into the newly created space (Bernstein, 1995; Cockett et al., 1994; Ruoslahti, 1996; Sato et al., 1994; Woodhouse et al., 1997). Among these steps, for tumor cells to invade, the first barrier to move is the matrix (Ruoslahti, 1996), which is composed of type IV collagen. Furthermore, MMP-2 and MMP-9 play a crucial role in the degradation of type IV collagen (Ruoslahti, 1996) and in the release of growth factors sequestered in the ECM, expose the binding sites for cell adhesion molecules, and activate growth factors and angiogenesis factors by cleaving their precursors. Therefore, they are important factors in instigating tumor cell growth, angiogenesis upregulation, and the triggering of tumor cell motility. Wild-Bode et al. (2001) reported that sublethal doses of irradiation enhance the migration and invasiveness of human malignant glioma cells by activation of MMP-2 and -9, and irradiation can enhance 9L rat glioma cell dissemination *in vivo* animal model using 9 L cells. Furthermore, Trog et al. (2006a,b) reported that temozolomide treatments alone, or combined chemo/radio treatment synergistically increase expression of MMP-2 and -9 at U87MG malignan glioma cells.

The covalent attachment of anticancer drugs to water-soluble polymers has several advantages, including: (1) since most anticancer drugs are hydrophobic, the use of water-soluble polymers solves the problem of their insolubility in water, (2) since the

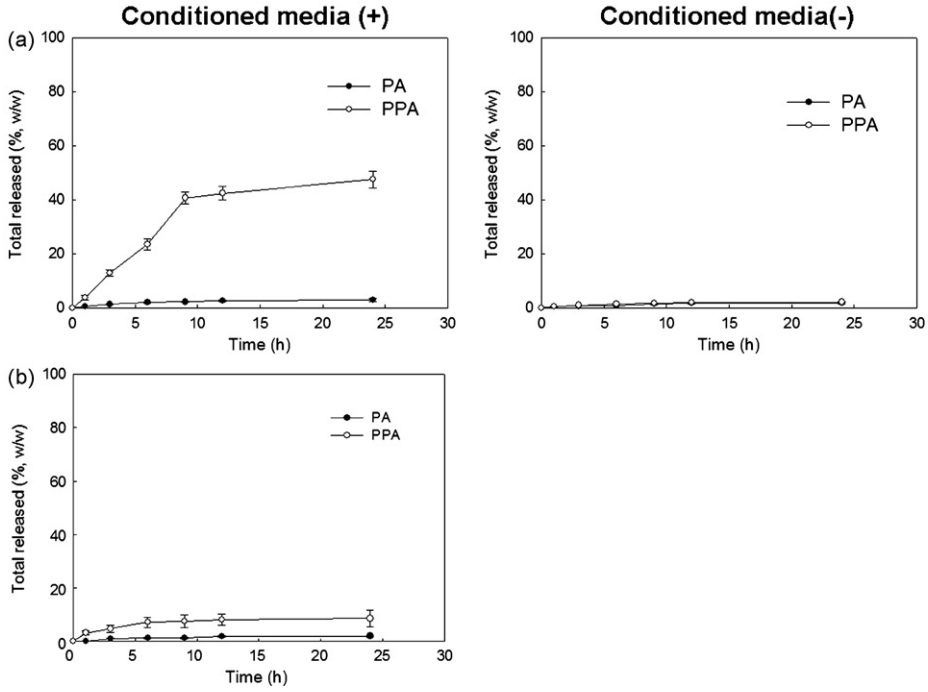

Fig. 4. ADR release from the drug–polymer conjugates in the absence or in the presence of conditioned media from the U87MG (a) and U251MG (b) cell culture. Each cell was confluently cultured on a 10 cm culture plate and the medium was replaced by 2 ml of serum-free DMEM. After 48 h of incubation, the conditioned media was collected and ultracentrifuged to remove the cell debris. To prepare it for use in the drug release test, the conditioned media was dialyzed against an ample amount of PBS.

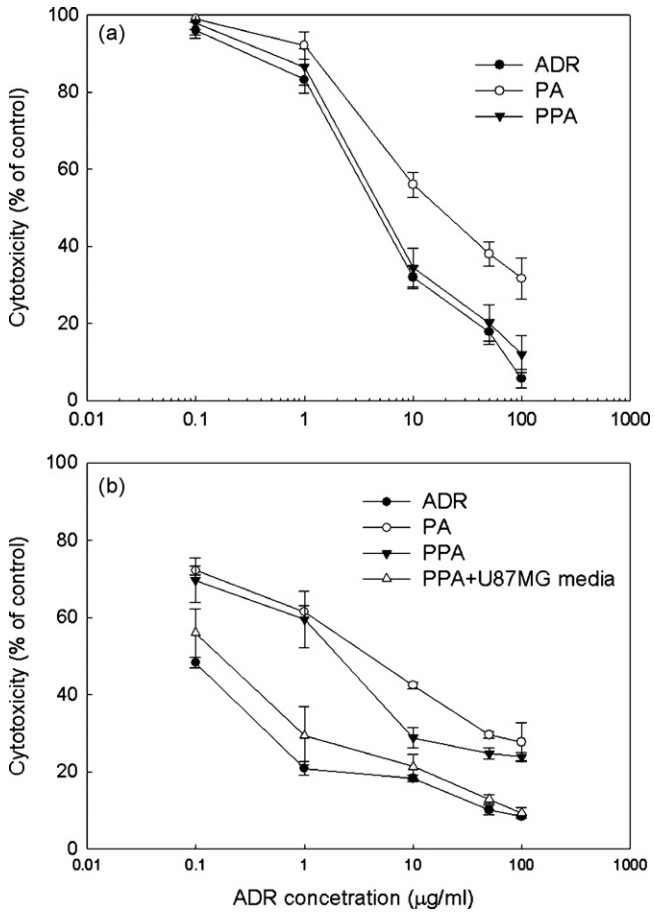

Fig. 5. Cytotoxicity of drug–polymer conjugates against U87MG cells (a) and U251MG cells (b). For comparison, PPA was dissolved in the conditioned media of U87MG cells and added to U251MG cells in a 96-well plate for the PPA + U87MG media.

molecular weights (MW) of polymers are much bigger than those of anticancer drugs, the half-life of polymer–drug conjugates in the circulation is much longer than that of the free drugs. The decreased total body clearance of the polymer–drug conjugates increase the amount of drug delivered to the tumor site (Duncan et al., 1992; Matsumura and Maeda, 1986; Nishikawa et al., 1996). Furthermore, it would be expected that the polymer–drug conjugates exhibit enhanced permeation and retention (EPR) to the tumor site, due to the fact that solid tumors have leaky blood vessels and poor lymphatic drainage (Maeda et al., 2000).

In our study, the MMP-cleavable PPA is composed of MPEG, a peptide, and ADR as shown in Fig. 2(c). ADR, one of the hydrophobic anticancer agents, has not been thought to have strong anticancer activity against glioma cells. However, the strong red color of ADR in UV-spectrophotometer makes it easy to perform the drug release experiment. MPEG generally prevents the uptake of conjugates by macrophages or the reticuloendothelial system and enhances the aqueous solubility of hydrophobic drugs. Recent investigation showed that PEGylation may enhance the drug delivery across the blood–brain barrier (BBB) through undetermined mechanism (Brigger et al., 2002). Although it was not proved whether or not PPA can be cross the BBB in this study, we found that the PEGylated ADR conjugates is ease to solubilize into aqueous solution and phosphated buffered saline (pH 7.4).

The peptide linker provides the targeting function, since MMP-2 and -9 are specifically overexpressed and excreted at the tumor tissues. MMP-induced cleavage of peptide occurs between P1 and P1′ in the peptide sequence (P3–P2–P1–P1′–P2′–P3′) (Turk et al., 2001). In our peptide sequence (Pro-Val-Gly-Leu-Ile-Gly), site between Gly and Leu (P1–P1′) is regarded as a cleavage site against MMP-2. As shown in Fig. 4, the non-cleavable conjugates (PA) are not responsive to the excretion of MMP from the tumor cells, because PA does not have a cleavable linker. From the results of the gelatin zymography (Fig. 3), we added conditioned media from the cell culture to the PA and PPA. As expected, PA was not responsive to the conditioned media of either the U87MG or U251MG cells, while ADR was released from PPA in a responsive manner to the

secretion of MMP from tumor cells. ADR or the ADR–peptide fragment might be released from the PPA conjugate according to the addition of conditioned media of U87MG cells (Fig. 4(a)). Practically, MPEG could not penetrate the dialysis tube, while the ADR or ADR–peptide fragment liberated from the PPA can move outside of the dialysis tube because its MW is smaller than the MWCO size of the dialysis tube. Because PA cannot be degraded by MMP, ADR cannot be released from the PA conjugates and the PA conjugates cannot penetrate the dialysis tube because the molecular weight of PA is bigger than the dialysis membrane. In the cell cytotoxicity test, the survivability of the U87MG tumor cells was similar to that of both ADR and PPA as shown in Fig. 5. However, PA was observed to be less cytotoxic to the tumor cells than ADR. These results indicate that ADR was released from PPA by the MMP-2 released from the U87MG cells, i.e. PPA has the potential to be used for MMP-responsive tumor targeting. Furthermore, the cytotoxicities of PA and PPA toward the U251MG cells were not significantly changed, while ADR showed dose-dependent cytotoxicity. When conditioned media from the U87MG cell culture was added to the U251MG viability test, the cytotoxicity of PPA against the U251MG cells was almost similar to that of ADR, indicating that ADR might be released by MMP (from the media of the U87MG cells) and that this ADR showed cytotoxicity. Because PPA with high ADR concentration demonstrated unexpected increase in cytotoxicity toward U251MG, further investigations would be necessary to clarify the cytotoxicity of these conjugates against the malignant glioma cells.

5. Conclusion

The present study demonstrates successful conjugation of MPEG and ADR with the MMP-cleavable peptide. The drug release and cytotoxicity tests also indicate the cleavability of ADR from PPA conjugate, responsive to the excretion of MMP-2 from malignant glioma cells. Taken together, the MMP-cleavable polymer–drug conjugates can be used as targeting carriers for the purpose of inhibiting the invasion of malignant glioma cells.

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