Human Monoclonal Antibody-Drug Conjugates in the Experimental Treatment of Malignant Gliomas

—Studies In Vitro and In Vivo—

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Abstract

The chemotherapeutic agents doxorubicin (DXR) and 4'-epi-doxorubicin were separately conjugated to the human monoclonal antibody CLNIgG, which binds strongly to human malignant glioma cells. The antibody-drug conjugates were more potent than the free drugs in killing human glioma cells *in vitro* and *in vivo* (subcutaneous glioma model). A biodistribution study using [14-¹⁴C]DXR-CLNIgG conjugate indicated that immunoconjugates delivered at least five times more DXR to glioma tissues than free DXR alone in nude mice without increasing the concentration in other tissues. In a nude rat intracerebral glioma model, intracarotid hyperosmolar perfusion to achieve blood-brain barrier (BBB) opening caused a 640% increase in the DXR-CLNIgG concentration in intracerebral tumor tissue. Human monoclonal antibody-drug conjugates in combination with BBB opening is a potential new approach to the treatment of malignant gliomas.

Key words: human monoclonal antibody, glioma, doxorubicin, 4'-epi-doxorubicin, conjugate, blood-brain barrier

Introduction

Malignant gliomas are the most common of all primary brain tumors, and are also among the most difficult to treat. Advances in the conventional treatment of malignant gliomas, including surgery,¹⁰⁾ radiation therapy, and systemic chemotherapy, have not essentially improved the prognosis of this disease for the past 20 years.^{12,16)} The 2-year survival rate for glioblastoma multiforme is still less than 20%.^{6,21)} A new therapy must therefore be developed.

Monoclonal antibody technology can provide reagents that selectively recognize cell surface antigens expressed by neoplastic tissues. However, in the area of immunotherapy, monoclonal antibodies are just beginning to fulfill the potential inherent in their great specificity for recognizing and selectively binding to antigens on tumor cells. The initial results of murine monoclonal antibodies in glioma therapy have been much less satisfactory, possibly due to the

immunogenicity of murine monoclonal antibodies, limitations to the efficacy of unmodified antibodies, and inadequate delivery or penetration across the blood-brain barrier (BBB).^{6,19)}

Although murine monoclonal antibodies are of value in the therapy of human diseases, their effectiveness is limited because rodent monoclonal antibodies, being xenogenic agents, are immunogenic and elicit a human immune response that neutralizes their therapeutic effect.¹⁹⁾ One solution to the problem of immunogenicity is to use human monoclonal antibodies. CLNIgG was produced by a human × human hybridoma, CLNH11, which was derived from uterine lymph node cells from a case of human uterine cancer.⁵⁾ CLNIgG was shown to react with human malignant gliomas as well as autologous cervical carcinomas,¹⁾ but reacted with neither normal adult and fetal brain tissues nor other, non-neural, tissues.⁸⁾

The limited efficacy of many unmodified monoclonal antibodies has led to an alternative approach, using these agents as carriers of cytotoxic substances.^{2,17)} More than 30 years ago, the use of chemotherapy to treat malignant brain tumors was

introduced as a supplement to surgery and radiation.²⁰ Experience has shown that systemic chemotherapy is not a cure for such tumors, because the lack of specificity for malignant cells leads to dose-limiting side effects and unacceptable toxicity. A promising area of investigation is the targeting of a cytotoxic agent to the tumor cells, which could be achieved by the preparation of antibody-drug conjugates.

Here we report an experimental study in the mouse using human monoclonal antibody conjugated with cytotoxic agents and osmotic opening of the BBB for antibody delivery into brain tumors.

Materials and Methods

I. Cell lines

Human malignant glioma (astrocytoma grade III and IV) cell lines, U373-MG and U87-MG, were described previously.^{15,18} The U373-MG cells were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum (FCS). The U87-MG cells were maintained in L-15 medium containing 10% FCS.

II. Monoclonal antibody and chemotherapeutic agent

The human monoclonal antibody CLNIgG, derived from human uterus cancer lymph node cells,⁵ is found to bind strongly to human malignant glioma cells.¹⁸ CLNIgG was provided by the Hagiwara Institute of Health (Kasai, Hyogo). The antigen recognized by CLNIgG is a membrane protein (226 kd) expressed by human malignant glioma cells.¹¹ Doxorubicin (DXR) and 4'-epi-doxorubicin (epi-DXR, a less cardiotoxic analogue of DXR⁷) were donated by Farmitalia Carlo Erba (Milano, Italy). The chemical structures are shown in Fig. 1.

III. Preparation of immunoconjugates

DXR or epi-DXR was conjugated to CLNIgG through a dextran bridge as previously described.²¹ Briefly, dextran-T₄₀ was oxidized with sodium periodate and incubated with DXR or epi-DXR for 20 hours at 4°C. The CLNIgG was then added and the mixture was left for a further 48 hours. The antibody-drug conjugates were separated from the unbound drug with a Sephadex G-100 (Pharmacia, Uppsala, Sweden) gel filtration apparatus. Protein analysis and spectroscopic analysis (OD_{495 nm}) showed that 38 molecules of the drug bound to one molecule of antibody. Normal human IgG (nIgG)-drug conjugate was prepared as a control in a similar way.

IV. Subcutaneous and intracerebral tumor models

The subcutaneous glioma model used 6-week-old male BALB/c athymic mice (nu/nu) inoculated subcutaneously in the back with 5×10^6 U87-MG glioma cells *via* a 26-gauge needle.⁴ The intracerebral glioma model used male Fischer 344/Jcl nude rats (run/run) (Clea Japan Inc., Tokyo) weighing 80–90 g which were injected with 25 μ l of U87-MG glioma cell suspension (5×10^5 cells) into the right hemisphere at a depth of 2.5 mm. The intracerebral tumors were identified on the 9th day after inoculation by magnetic resonance (MR) imaging with a JNM-IM60 imaging system (6.3 T; JEOL, Tokyo).

V. Pharmacological activities of immunoconjugates *in vitro*

The immunoreactivity was measured before and after drug conjugation by determining the binding to glioma cells with an enzyme-linked immunosorbent assay (ELISA). The cytotoxic activity of the antibody-drug conjugates was assessed by a ³H-thymidine incorporation assay. Briefly, 5×10^5

Fig. 1 Chemical structures of epi-DXR (left) and DXR (right).

U373-MG cells/well were exposed to the serially diluted immunoconjugates for 4 hours at 37°C. The cells were then washed twice. Subsequently, 1 μ Ci of 3 H-thymidine (Amersham, Buckinghamshire, U.K.) was added to each well followed by incubation for 18 hours. The cells were harvested, and the incorporation of 3 H-thymidine was determined with a β -counter (Aloka, Tokyo).

VI. Efficacy of immunoconjugates *in vivo*

Seven days after tumor inoculation, the nude mice that had developed a palpable subcutaneous tumor were selected for treatment with intraperitoneal injection of one of the following: a) phosphate-buffered saline (PBS), b) epi-DXR-CLNIgG conjugate, or c) epi-DXR alone at 3-day intervals for a total of four times. Each treatment group contained six mice. Each animal received a total equivalent dose of 1 mg/kg of epi-DXR or 8 mg/kg of CLNIgG, or both. Tumor growth was determined every 3rd day by measuring the short (a) and long (b) dimensions of the tumors with calipers. The tumor volumes were calculated by the following formula:

$$\text{tumor volume} = (a^2 \cdot b) / 2$$

The differences in tumor volumes between experimental groups were analyzed by Wilcoxon's rank test. 31 P-MR spectra were examined *in vivo* to measure the energy metabolism of tumors using a JSM-SMR 270 spectrometer system (JEOL). Twelve hours after the first treatment, the subject animals were anesthetized by nembutal and fixed in the MR apparatus so that the tumor mass was located on a four-turn surface coil of 1.0 cm in diameter, providing a resonant signal of 31 P from the tumor.

VII. Biodistribution of immunoconjugates

The tissue distributions of DXR-CLNIgG conjugates and of DXR after intraperitoneal injection were compared in nude mice bearing U87-MG tumors. Briefly, 1 μ Ci of [14 C]DXR (Amersham) or 2 μ Ci of [14 C]DXR-CLNIgG was injected intraperitoneally into seven mice with an average tumor weight of 400 mg. Twenty-four hours after the injection, the mice were sacrificed under nembutal anesthesia. Major tissues were dissected, weighed, and dissolved in 4 ml of Soluene 350 (Packard, Meriden, Conn., U.S.A.). The dissolved tissues were then counted on a β -counter using Hionic-Fluor (Packard) as the liquid scintillation cocktail. The results were expressed as a percentage of the injected dose per gram of tissue (%ID/g).

VIII. Osmotic BBB opening

The delivery of DXR-CLNIgG conjugates to intracerebral tumors after BBB opening was evaluated in F₃₄₄ nude rats bearing human glioma xenografts 10 days after tumor inoculation. The technique of BBB disruption in rats was according to Neuwelt *et al.*¹³⁾ All rats underwent right carotid artery exposure and were infused *via* a carotid artery catheter with physiological saline in control studies or with 20% mannitol (1 mg/kg) in BBB opening studies. 2 μ Ci of [14 C]DXR-CLNIgG was administered *via* the same catheter immediately after saline or mannitol infusion over 1 minute. Thirty minutes after conjugate administration, the animals were sacrificed under nembutal anesthesia and the tumor and 1–2 mm of brain tissue immediately surrounding the tumor, the contralateral hemisphere of the brain, and other organs and tissues were dissected for [14 C]DXR-CLNIgG determination as described above.

Results

I. Pharmacological activities of immunoconjugates *in vitro*

CLNIgG conjugated with 38 molecules of epi-DXR or DXR retained about 90% of its immunoreactivity after conjugation measured by ELISA. The cytotoxic activity of the immunoconjugates was monitored by an assay measuring 3 H-thymidine incorporation, since epi-DXR and DXR are known to intercalate with deoxyribonucleic acid and cause cell cycle arrest in the G₂ phase. The epi-DXR-CLNIgG

Fig. 2 Cytotoxicity of epi-DXR-CLNIgG conjugate (●), epi-DXR (○), CLNIgG (▲), and CLNIgG with epi-DXR (△). The values are the means of triplicate assessments.

Fig. 3 Growth curves of human glioma xenografts in nude mice after drug treatments (arrows) with PBS (■), epi-DXR (○), and epi-DXR-CLNIgG (●). Bars indicate $\pm$ SE of the mean. * $p < 0.01$ vs. PBS control group, ** $p < 0.001$ vs. epi-DXR group.

Fig. 4 ^{31}P -MR spectra of gliomas in three mouse groups treated with PBS (upper), epi-DXR-CLNIgG (middle), and epi-DXR (lower). I: β -ATP, II: α -ATP and α -ADP, III: γ -ATP and β -ADP, IV: phosphocreatine, V: inorganic phosphate, VI: phosphomonoesters.

conjugate was found to be 11 times more potent than free epi-DXR in killing U373-MG glioma cells (Fig. 2). The 50% inhibitory concentration (IC_{50}) of epi-DXR-CLNIgG was 4.1×10^{-9} M, while that of free epi-DXR was 4.4×10^{-8} M. A mixture of CLNIgG antibody and epi-DXR did not demonstrate a significantly increased cytotoxic efficacy (IC_{50} 4.1×10^{-8} M). Epi-DXR-nIgG (IC_{50} 5.0×10^{-8} M) and epi-DXR-dextran (IC_{50} 5.4×10^{-8} M) conjugates did not achieve greater inhibition of glioma cell growth than epi-DXR.

II. Efficacy of immunoconjugates *in vivo*

Figure 3 shows the efficacy of the various agents in suppressing tumor growth. Epi-DXR-CLNIgG clearly achieved the most favorable antitumor effects. A significant tumor growth difference ($p < 0.01$) between the epi-DXR-CLNIgG and PBS control groups was observed as early as 6 days after the start of treatment. Free epi-DXR achieved only non-significant inhibition of tumor growth. Epi-DXR-CLNIgG showed significant ($p < 0.001$) inhibition of tumor growth at a treatment/control volume (T/C) of 0.42, as compared with a T/C of 0.85 in the epi-DXR group, at the end of the study (day 24). The ^{31}P -MR spectra of the tumors are shown in Fig. 4. Several peaks appear in these spectra, in descending order of resonance frequency: β -adenosine triphosphate (ATP), α -ATP and α -adenosine diphosphate (ADP), γ -ATP and β -ADP, phosphocreatine, inorganic phosphate, and phosphomonoesters. After treatment with epi-DXR-CLNIgG, the intensity of the ATP peaks had decreased markedly, but no such change occurred in the epi-DXR group.

III. Biodistribution of immunoconjugates

The most striking finding was the greater deposition of DXR in the tumor tissues by DXR-CLNIgG conjugates compared to the free drug (Table 1). Only 0.77% of the total $[14\text{-}^{14}\text{C}]\text{DXR}$ injected was present in each gram of tumor tissue, whereas 3.9% of the total $[14\text{-}^{14}\text{C}]\text{DXR-CLNIgG}$ injected was present in each gram of tumor. Thus the immunoconjugates achieved a delivery of five times as much DXR to the target site. Another notable point was that tumor/tissue ratios of $[14\text{-}^{14}\text{C}]\text{DXR-CLNIgG}$ were nine times those of $[14\text{-}^{14}\text{C}]\text{DXR}$ in the bone marrow, eight times in the intestines, and seven times in the heart.

IV. Osmotic BBB opening

Following intracarotid perfusion with mannitol, there was a 640% increase in the delivery of $[14\text{-}^{14}\text{C}]\text{DXR-CLNIgG}$ into the intracerebral glioma tissues

Table 1 Distribution of [^{14}C]DXR-CLNIgG in human glioma-grafted nude mice

Tissue	Specific tissue activity (%ID/g)		Tumor/tissue ratio of radioactivity (cpm/g tumor/cpm/g tissue)	
	[^{14}C]DXR-CLNIgG	[^{14}C]DXR	[^{14}C]DXR-CLNIgG	[^{14}C]DXR
Tumor	3.904 $\pm$ 0.663	0.772 $\pm$ 0.044	1.0	1.0
Blood	1.350 $\pm$ 0.333	0.694 $\pm$ 0.083	2.935 $\pm$ 0.265	1.118 $\pm$ 0.075
Stomach	1.039 $\pm$ 0.052	1.078 $\pm$ 0.321	3.752 $\pm$ 0.583	0.776 $\pm$ 0.303
Liver	1.517 $\pm$ 0.116	1.379 $\pm$ 0.102	2.563 $\pm$ 0.290	0.562 $\pm$ 0.071
Spleen	2.782 $\pm$ 1.180	2.882 $\pm$ 0.515	1.617 $\pm$ 0.472	0.274 $\pm$ 0.058
Pancreas	0.843 $\pm$ 0.208	2.202 $\pm$ 0.772	4.823 $\pm$ 1.247	0.397 $\pm$ 0.128
Kidney	2.355 $\pm$ 0.529	3.175 $\pm$ 0.411	1.694 $\pm$ 0.207	0.243 $\pm$ 0.044
Small intestine	1.030 $\pm$ 0.210	1.774 $\pm$ 0.386	3.835 $\pm$ 0.525	0.450 $\pm$ 0.114
Large intestine	1.095 $\pm$ 0.667	2.013 $\pm$ 0.086	3.405 $\pm$ 1.274	0.384 $\pm$ 0.037
Heart	0.445 $\pm$ 0.156	0.736 $\pm$ 0.352	9.374 $\pm$ 2.893	1.272 $\pm$ 0.722
Lung	1.117 $\pm$ 0.439	1.278 $\pm$ 0.565	4.013 $\pm$ 1.998	0.676 $\pm$ 0.261
Bladder	1.407 $\pm$ 0.234	2.629 $\pm$ 0.170	2.791 $\pm$ 0.363	0.294 $\pm$ 0.008
Muscle	0.512 $\pm$ 0.052	0.584 $\pm$ 0.140	7.649 $\pm$ 1.281	1.561 $\pm$ 0.450
Bone marrow	0.602 $\pm$ 0.103	1.455 $\pm$ 0.276	5.387 $\pm$ 1.534	0.545 $\pm$ 0.122

Values are mean $\pm$ SE of three to four mice at 24 hours after injection of [^{14}C]DXR-CLNIgG or [^{14}C]DXR.

Table 2 [^{14}C]DXR-CLNIgG concentration in nude rats after BBB opening

Tissue	[^{14}C]DXR-CLNIgG (%ID/g)	
	BBB opening (+)	BBB opening (-)
Brain tumor	6.320	0.982
Brain around tumor	3.841	0.065
Brain contralateral to tumor	0.177	0.010
Stomach	0.924	0.902
Spleen	1.180	1.246
Liver	1.809	1.855
Kidney	1.223	1.143
Intestine	1.049	0.931
Heart	0.573	0.648
Lung	0.865	1.004
Bone marrow	0.746	0.786

(Table 2). BBB opening also increased the delivery of immunoconjugates to the brain around the tumor and to the contralateral brain tissues. The highest concentration of immunoconjugates was in the tumor tissues. The distribution of immunoconjugates in non-neural tissues showed no difference between the BBB opening and physiological saline control groups.

Discussion

We prepared immunoconjugates by coupling CLNIgG to DXR or epi-DXR through a dextran

bridge. The cell surface binding of the resultant antibody-drug conjugates to human glioma cells was very efficient, and was similar to that of the original antibody. Epi-DXR-CLNIgG was 11 times more potent than free epi-DXR against glioma cells. Since free CLNIgG in the absence of lymphocytes or of complement cannot exert direct cytotoxicity against glioma cells (Fig. 2), and since it lacks synergy with epi-DXR, we interpret the present finding of increased cytotoxicity of epi-DXR when conjugated to CLNIgG as an indication that the antibodies have directed the drug to the glioma cells. Alternatively, epi-DXR conjugated through a dextran bridge to protein may have caused a non-specific uptake by tumor cells. However, this was not substantiated in our experiments in which non-specific epi-DXR-nIgG and epi-DXR-dextran conjugates achieved no better cytotoxicity than free epi-DXR. Thus, our results indicate that the cytotoxic activity of our immunoconjugates was mediated by the specificity of the CLNIgG antibody.

The results of treatment with immunoconjugates *in vivo* demonstrate that epi-DXR-CLNIgG is very effective in suppressing the growth of established human glioma xenografts in nude mice (T/C 0.42). This improvement was significantly ($p < 0.001$) better than that resulting from treatment with epi-DXR (T/C 0.85). ^{31}P -MR spectrum studies further showed that ATP peaks decreased after treatment with epi-DXR-CLNIgG, indicating that the energy metabolism and cell growth of the tumors were inhibited.⁹⁾

Our biodistribution study provided further

evidence that the DXR-CLNIgG conjugate can deliver at least five times more DXR (3.9%ID/g) to the tumor tissue than the free form (0.77%ID/g). Moreover, less DXR-CLNIgG than DXR accumulated in the bone marrow and heart tissues, indicating that antibody-drug conjugates effectively increase the therapeutic index of cytotoxic agents. These results are consistent with the tumor suppression observed *in vivo*.

A major potential difficulty with immunoconjugate therapy is the large size of the molecules which limits the transvascular diffusion into brain tumor tissue through the BBB.⁶⁾ Transient hyperosmolar disruption of the BBB has been performed safely in animals and in man, generally in studies involving the delivery of chemotherapeutic agents to the central nervous system.¹¹⁾ Some evidence exists that this technique can also be used to deliver large molecules such as antibodies.¹³⁾ In the present study, we evaluated the delivery of antibody-drug conjugates into brain tumors and the immediately adjacent brain tissues after osmotic disruption of the BBB in nude rats. Our findings demonstrate several points. The delivery of DXR-CLNIgG conjugates into intracerebral tumors is increased by 640% after transient BBB opening with the hyperosmolar agent mannitol administered by the intracarotid route. After BBB opening, the immunoconjugate concentration in the cerebral tissues around the tumor was also increased. The intracerebral tumor tissue, however, contained more immunoconjugates than the immediately surrounding tissues. The ipsilateral nature of BBB opening has been stressed by some authors.¹²⁾ In our study, increased delivery of DXR-CLNIgG occurred in bilateral hemispheres of the brain following BBB disruption, although it was much higher in the ipsilateral hemisphere. Bullard *et al.*³⁾ found similar findings in delivering monoclonal antibodies to the normal rat brain after BBB opening. Indeed, variability in distribution based on the circle of Willis appears to be the rule rather than the exception. Since there are few receptors for the Fc portion of immunoglobulins in the brain,¹⁴⁾ the presence of immunoconjugates in the brain tissue may be the result of nonspecific binding to the Fc receptors on the brain cell membranes. Studies are in progress to utilize Fab fragments to reduce this effect.

Although our results show that further investigations are required for clinical trials, the present studies indicate that the use of human monoclonal antibody-drug conjugates in combination with BBB opening is a promising therapeutic strategy which optimizes the use of these novel agents for the treatment of malignant gliomas.

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