LABORATORY INVESTIGATION

Effects of Hydroxychloroquine Co-administered with Chemotherapeutic Agents on Malignant Glioma Cell Lines : *in vitro* Study

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Objective : Anti-malaria drugs may modulate tumor resistance to chemotherapeutic agents, but it has not been proven effective in the treatment of malignant gliomas. The aim of this study was to determine whether adequate pre-clinical data on co-administration of chemotherapeutic agents with anti-malaria drugs on malignant cell lines could be obtained that would warrant its further potential consideration for use in a clinical trial for malignant gliomas.
Methods : Two malignant glioma cell lines (U87MG, T98G) were treated with chemotherapeutic agents alone or with anti-malaria drugs. Cells were incubated with drugs for 4 days. Following the 4-day incubation, drug sensitivity assays were performed using 3-(4,5-dimethyl-2-thiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay following optimization of experimental conditions for each cell lines and cell viability was calculated.
Results : In all of four chemotherapeutic agents(doxorubicin, vincrisitne, nimustine, and cisplatin), the cell viability was found to be markedly decreased when hydroxychloroquine was co-administered on both U87MG and T98G cell lines. The two way analysis of variance(ANOVA) yielded a statistically significant two-sided p-value of 0.0033(doxorubicin), 0.0005(vincrisitne), 0.0007(nimustine), and 0.0003(cisplatin) on U87MG cell lines and 0.0006(doxorubicin), 0.0421(vincrisitne), 0.0317(nimustine), and 0.0001(cisplatin) on T98G cell lines, respectively. However, treatment with chloroquine and primaquine did not induce a decrease in cell viability on both U87MG and T98G cell lines.
Conclusion : Our data support further consideration of the use of hydroxychloroquine prior to systemic chemotherapy to maximize its tumoricidal effect for patients with malignant gliomas.

KEY WORDS : Anti-malarial drugs · Hydroxychloroquine · Chemotherapy · Multidrug resistance · Malignant glioma.

Introduction

Many ongoing studies have explored new therapeutic approaches to improve the outcome for patients with malignant gliomas in the recent years. Even though, systemic chemotherapy is considered as an principal adjuvant treatment to surgical resection in the management of malignant gliomas, intrinsic or induced drug resistance is thought to be one of the obstacles in chemotherapy[2,9,26].

The mechanisms underlying this drug resistance have been studied, leading to the characterization of genes capable of conferring resistance to chemotherapeutic drugs. Among these genes, the multi-drug resistance type 1 (*mdr1*) gene has been of particular interest because its over-expression can lead to resistance to cytotoxic drugs such as anthracyclines, vinca alkaloids, and epipodophyllotoxins by the P-glycoprotein (P-gp)[1,3,5,7-10,14,16,21,22-24,32]. Increased levels of P-gp are common in cancer cells. Moreover, levels of the glycoprotein can be increased after chemotherapy, when the tumor becomes refractory to treatment. P-gp encoded by *mdr1* gene functions as membrane-bound transporters, mediating ATP-dependent efflux of various structurally and functionally unrelated substrates from the cell[10,24,25]. Some chemosensitizing agents have been shown to overcome *mdr1* gene phenotype in experiments. Recently, anti-malarial drugs have raised considerable interest because of its anti-carcinogenic properties and its

ability to inhibit tumor development and virus replication[31]. Lagneaux and colleagues reported that hydroxychloroquine induced an early apoptosis in B-chronic lymphocytic leukemia cells[18]. Inaba et al reported that an antimalarial drug, especially quinacrine, exhibited therapeutic synergism in combination with vincristine in resistant leukemia-bearing mice[13]. In the literature, however, few reports are available pertaining to the experimental or clinical trial of chemosensitizing agents prior to systemic chemotherapy in malignant gliomas.

The aim of this study was to determine whether adequate pre-clinical data on co-administration of chemotherapeutic agents with anti-malaria drugs could be obtained that would warrant its further consideration for use in a clinical trial for malignant gliomas.

Materials and Methods

Cell lines

Human glioblastoma cell lines, U87MG and T98G, were obtained from the American Type Culture Collection (Manassas, VA). All cells were grown as monolayers in Dulbecco's modified Eagle's medium (Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum(FBS) (ICN Biomedicals, Inc., Costa Mesa, CA), penicillin, and streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Drugs

All drugs were obtained from commercial sources : doxorubicin(adriamycin; Farmitalia Carlo Erba Ltd, Italy), vincrisitne(oncovin; Eli Lilly, USA), nimustine(ACNU; Sankyo Co., LTD, Japan), cisplatin(platinol; Bristol-Myers Squibb, USA), hydroxychloroquine(oxiklorin; Orion Pharmaceutica, Finland), chloroquine(aralen; Sanofi Winthrop, USA), primaquine(primaquine; Sanofi Winthrop, USA). The chemotherapeutic agents were tested over the following concentration ranges : doxorubicin 0.01, 0.1, 1, and 10μg/ml; vincristine 0.005, 0.05, 0.5, and 5μg/ml; nimustine 0.005, 0.05, 0.5, and 5μg/ml; cisplatin 0.005, 0.05, 0.5, and 5μg/ml. The anti-malaria drugs were employed with the following concentration ranges : hydroxychloroquine 15μg/ml and 30μg/ml; chloroquine 20μg/ml and 40μg/ml; primaquine 1.5μg/ml and 3μg/ml. All drugs were dissolved or diluted with phosphate buffered saline(PBS) and prepared immediately prior to use.

3-(4,5-dimethyl-2-thiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay

Single cell suspensions were obtained by pipet disaggregation of the floating cell line or by trypsinization of monolayer cultures, and cell counts were performed using a hemocytometer. The assay was dependent on the cellular reduction of MTT

(Sigma Chemical Co., St.Louis, MO) by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically. Cells were plated in 180μℓ medium at the appropriate seeding density into 96 well microtiter plates after preliminary cell growth studies using MTT assay so that untreated cells were in exponential growth phase at the time of initial harvest and at the end of the 4-day incubation. Cell number per microtiter well was proportional to the absorbance of the solubilized formazan. To each well 20μℓ of 10x drug in PBS was added, with PBS added to control wells. Cells were incubated with or without drug for 4days, allowing sufficient time for cell replication, drug induced cell death, and loss of enzyme activity, which generate the formazan product. Following the 4day incubation, 0.1mg (50μℓ of 2mg/ml) of MTT solution was added to each well, and the plates incubated at 37°C for 4h. The microtiter plates were centrifuged at 500g for 5min, 200μℓ supernatant removed from plates leaving about 30μℓ residual medium in each well. Care had to be taken not to disturb the formazan crystals at the bottom of the wells. MTT formazan crystals were resolubilized by adding 130μℓ 100% dimethylsulfoxide (DMSO) to each well. Plates were then placed on a shaker for 10min. The plates were read immediately at 540nm on a scanning multiwell ELISA automatic spectrophotometer recorder (Behring ELISA Processor II, Germany). Cell survival(viability) was calculated by the following formula :

$$\% \text{ viability} = 100 \times \frac{\text{Average absorbance of tested group-baseline absorbance}}{\text{Average absorbance of control group-baseline absorbance}}$$

Data points in Fig. 1, 2, and 3 represent cell survival 4days after plating and drug exposure measured by the MTT assay for doxorubicin, vincrisitne, nimustine, and cisplatin for 3 different anti-malaria drugs on 2 different malignant glioma cell lines.

Statistical analysis

Cell viability between anti-malaria drugs-treated and control cells (chemotherapeutic agents only) was analyzed using the two way analysis of variance (ANOVA). A probability value of less than 0.05 was considered significant. All statistical analyses were performed with commercially available software (SAS, version 6.12, SAS Inc., Cary, NC).
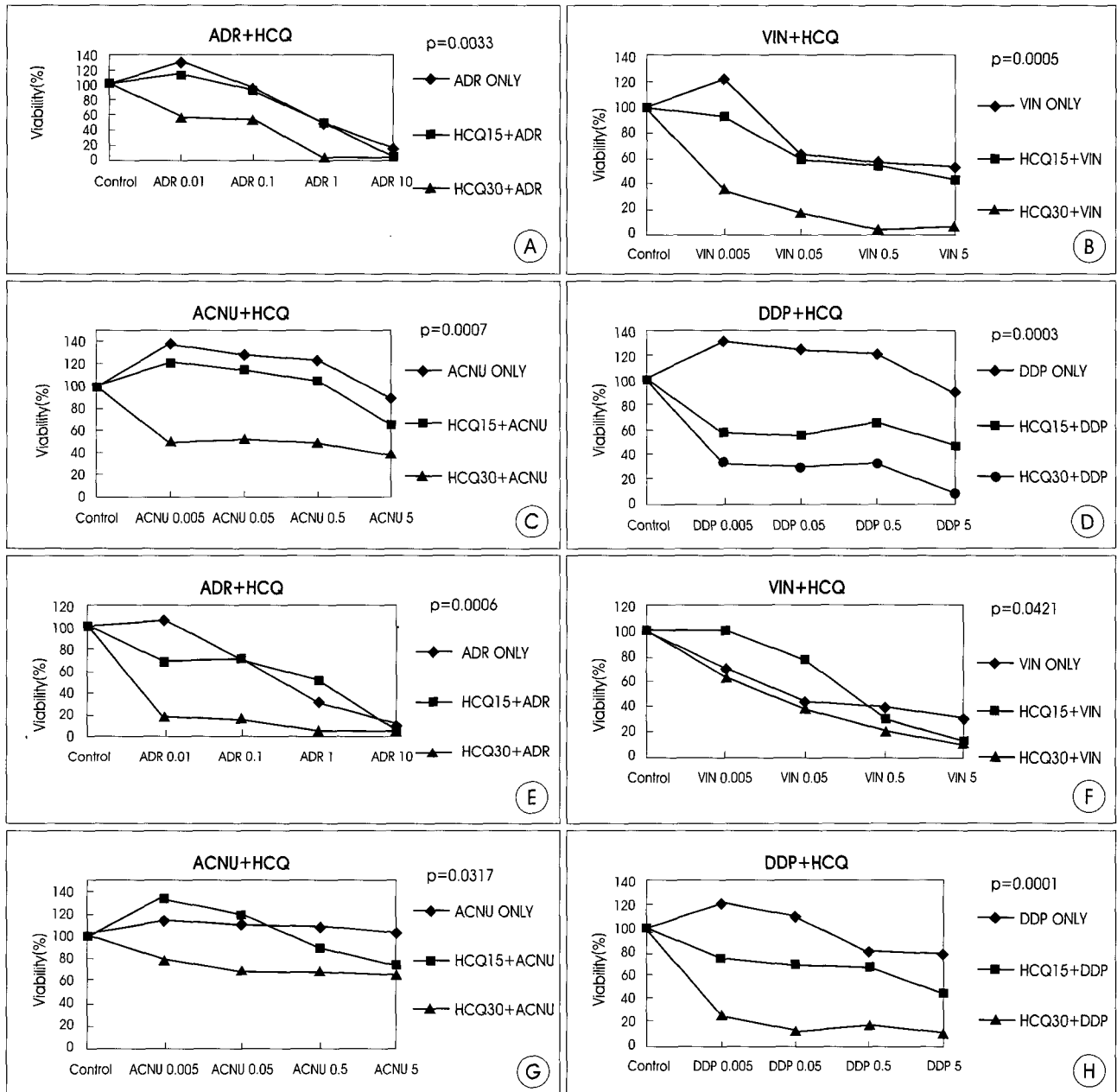
Results

Effects of hydroxychloroquine co-administered with chemotherapeutic agents

To evaluate the effects of hydroxychloroquine co-administered

with chemotherapeutic agents, hydroxychloroquine-treated and control cells(chemotherapeutic agents only) were analyzed for cell survival. Treatment with hydroxychloroquine was found to cause cell viability to decrease significantly as the concentration of hydroxychloroquine increased on both U87MG and T98G cell line. (Fig. 1A,B,C,D) shows these characteristic changes for hydroxychloroquine co-administered with chemotherapeutic agents in cell survival.

In all of four chemotherapeutic agents(doxorubicin, vincrisitne, nimustine, and cisplatin), cell viability was found to be markedly decreased. The two way ANOVA yielded a two-sided p-value of 0.0033(doxorubicin), 0.0005(vincrisitne), 0.0007(nimustine), and 0.0003(cisplatin) on U87MG cell line, respectively, which indicated that the effect of hydroxychloroquine co-administered with chemotherapeutic agents was statistically significant.
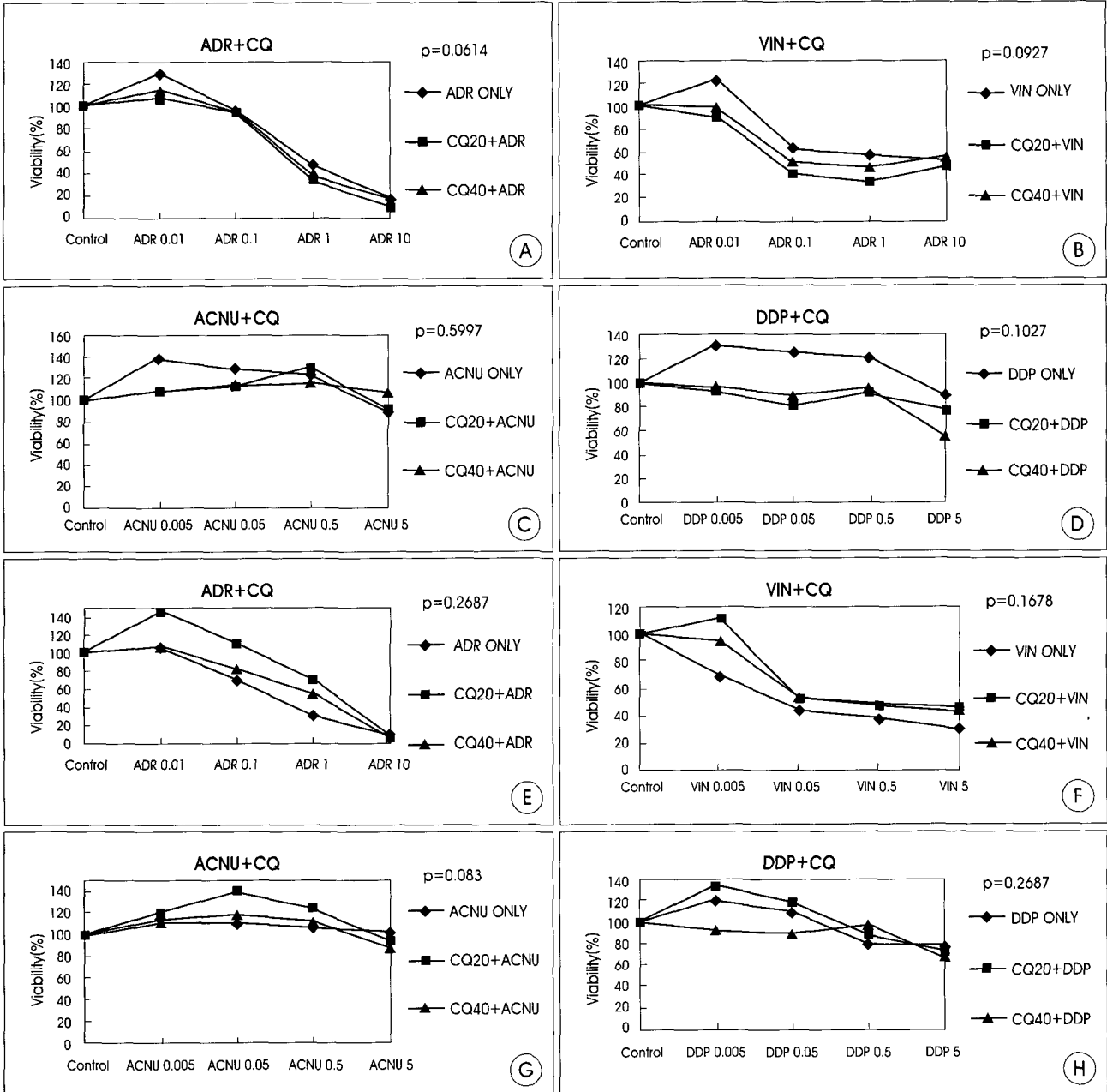

Fig. 1. Graph showing the effect of treatment with hydroxychloroquine. A~D, changes for hydroxychloroquine co-administered with chemotherapeutic agents on U87MG cell lines. E~F, changes for hydroxychloroquine co-administered with chemotherapeutic agents on T98G cell lines. In all of four chemotherapeutic agents(doxorubicin, vincrisitne, nimustine, and cisplatin), cell viability was found to markedly decrease. The two way ANOVA yielded a two-sided p-value of 0.0033(doxorubicin), 0.0005(vincrisitne), 0.0007(nimustine), and 0.0003(cisplatin) on U87MG cell lines and 0.0006(doxorubicin), 0.0421(vincrisitne), 0.0317(nimustine), and 0.0001(cisplatin) on T98G cell lines, respectively. ADR, doxorubicin; VIN, vincristine; ACNU, nimustine; DDP, cisplatin; HCQ, hydroxychloroquine.

To determine if similar effect occurred, we used a T98G cell line. (Fig. 1E, F, G, H) also shows similar characteristic changes in cell survival. In control cells (chemotherapeutic agents only), there was a high cell viability, whereas treatment with hydroxychloroquine decreased the cell viability. The two way ANOVA yielded a statistically significant p-value of 0.0006(doxorubicin), 0.0421(vincrisitne), 0.0317(nimustine), and 0.0001(cisplatin), respectively.

The effect of chemotherapeutic agents followed a concentration-dependent pattern, but the cell viability was markedly diminished at its lowest level of each anti-cancer drug on both U87MG and T98G cell line.

Effects of chloroquine and primaquine co-administered with chemotherapeutic agents
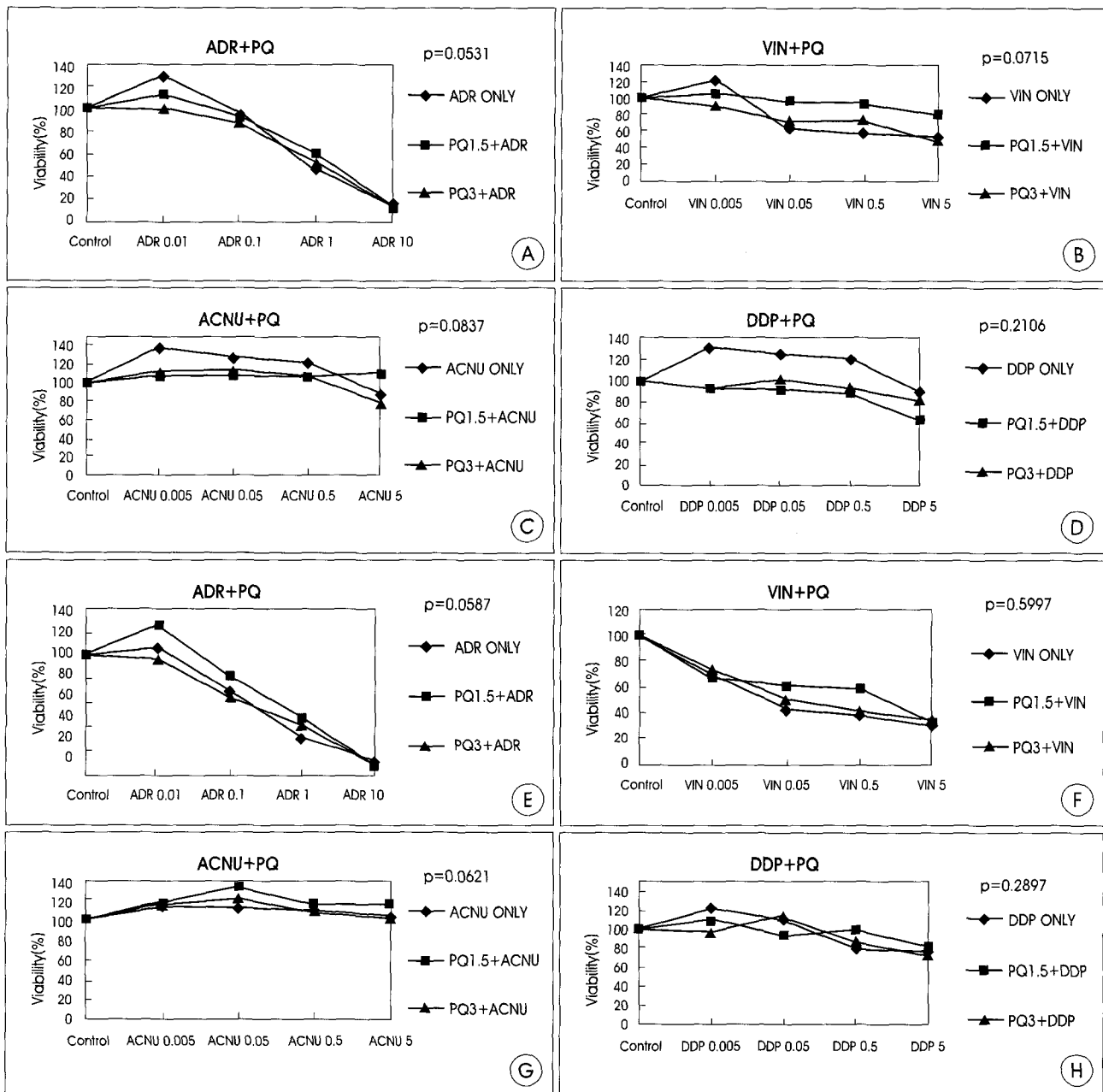
Experiments were also performed to determine whether

Fig. 2. Graph showing the effect of treatment with chloroquine. A~D, changes for chloroquine co-administered with chemotherapeutic agents on U87MG cell lines. E~F, changes for chloroquine co-administered with chemotherapeutic agents on T98G cell. Chloroquine was not found to decrease the cell viability on glioma cell lines. The two way ANOVA showed no statistical significance. In chloroquine-treated cells with doxorubicin only, there was a trend approaching statistical significance(p=0.0614). ADR, doxorubicin; VIN, vincristine; ACNU, nimustine; DDP, cisplatin; CQ, chloroquine.

treatment with other anti-malaria drugs(chloroquine, primaquine) would have a similar effect on both U87MG and T98G cell line.

Unlike the effect of treatment with hydroxychloroquine, chloroquine was not found to decrease the cell viability on glioma cell line. The two way ANOVA showed no statistical significance(Fig. 2). In chloroquine-treated cells with doxorubicin only(Fig. 2A), there was a trend approaching statistical

significance(p=0.0614). Results of cell viability for chloroquine are shown in Fig. 2.

Primaquine was also used to compare the cell viability between anti-malaria drugs-treated and control cells. Treatment with primaquine showed a similar effect to chloroquine on both U87MG and T98G cell line. The two way ANOVA showed no statistical significance(Fig. 3). In primaquine-treated cells with doxorubicin on both U87MG and T98G

Fig. 3. Graph showing the effect of treatment with primaquine. A~D, changes for primaquine co−administered with chemotherapeutic agents on U87MG cell lines. E~F, changes for primaquine co−administered with chemotherapeutic agents on T98G cell. Treatment with primaquine showed a similar effect to chloroquine on both U87MG and T98G cell line. The two way analysis of variance showed no statistical significance. In primaquine−treated cells with doxorubicin on both U87MG and T98G cell, there was a trend approaching statistical significance(p=0.0531, 0.0587, respectively). ADR, doxorubicin; VIN, vincristine; ACNU, nimustine; DDP, cisplatin; PQ, primaquine.

cell lines(Fig. 3A, E), there was a trend approaching statistical significance(p=0.0531, 0.0587, respectively). Results of cell viability for primaquine are shown in Fig. 3.

Discussion

This study paradigm has demonstrated that hydroxychloroquine induced a dramatic tumoricidal effect of anti-cancer drugs on malignant glioma cell lines in vitro. Treatment with hydroxychloroquine produced a marked decrease in glioma cell viability. The mechanisms in this model are not known but, multi-drug resistance likely plays a significant role[3,7-10,14,19-21,23-25].

Biedler and colleagues first described the phenomenon of in vitro multi-drug resistance in the 1970s[5]. Cells selected for resistance to one drug demonstrated cross resistance to other structurally and functionally unrelated compounds. The drugs that constitute the multi-drug resistance include the anthracyclines, the vinca alkaloids, and the epipodophyllotoxins. The multi-drug resistance phenotype is due apparently to an important energy-dependent reduction in the intracellular accumulation of cytotoxic drugs in resistant cells, caused by an outward efflux[10]. The resistant cell lines overexpress a transmembrane glycoprotein (P-glycoprotein, P-gp), which is encoded by the *mdr1* gene in human cells. It is commonly found in carcinomas of the kidney, liver, adrenal glands, and colon, and it is presumed that the increased resistance of these carcinomas to cytostatic treatment is due to the expression of P-gp[28]. In normal tissue the highest P-gp levels are found in the adrenal glands, capillary endothelium of the brain, and the kidney[11]. The high degree of chemoresistance in colorectal carcinoma is well known in the oncological community[17]. Veneroni and colleagues reported that a strong P-gp expression might serve as a negative prognostic marker for the responsiveness of breast cancer to chemotherapy[30]. Dietzmann et al. demonstrated a positive correlation between the fraction of P-gp-positive cells stained with the monoclonal antibody JSB1 and tumor grading in glioma[6].

Several drugs affected by *mdr1* gene have made it an attractive candidate to explain the phenomenon of multi-drug resistance, whereby a tumor becomes refractory to drugs to which it was never exposed. Some chemosensitizing agents have been shown to overcome mdr1 phenotype in experiments. Among them were calcium channel blockers such as verapamil and quinine, calmodulin inhibitors such as phenothiazines, the triparanol analogs tamoxifen, and cyclosporine[21,25,29]. These compounds also act mainly through inhibition of the drug pumping by P-gp. Clinical studies of such chemosensitizing agents given in combination with chemotherapy have been performed in patients with hematologic malignancies or solid tumors[25]. Many of these studies attempted to achieve plasma concentration of these agents. However, the significance of the plasma concentration remains unclear, because we have virtually no knowledge of the concentrations of these chemosensitizing agents and anti-cancer drugs in tumors.

Recently, anti-malaria drugs have raised considerable interest because of its anti-carcinogenic properties and its ability to inhibit virus replication and tumor development[31]. Lagneaux and colleagues reported that hydroxychloroquine induced an early induction of apoptosis in B-chronic lymphocytic leukemia cells[18]. In the literature, few reports are available pertaining to the effect of treatment with anti-malarial drugs prior to chemotherapy.

A significant finding from our experiment was that treatment with hydroxychloroquine caused the cell viability of malignant glioma to decrease significantly as their concentration increased. The effect of chemosensitizing agents on tumor cells has been postulated to result from impairing the pumping function of P-gp. The presumed mechanism in our model is to bind P-gp and competitively interact with chemotherapeutic agents-binding site of P-gp. As a result, anti-malria drugs enhance the intracellular concentration of cytotoxic drugs. However, other mechanisms for reversal of multi-drug resistance cannot be excluded. For instance, it was reported that promoting a redistribution of the chemotherapeutic agent within the resistant cell allowed the drug to reach its targets[4]. Also, the level of the protein kinase C responsible for the phosphorylation of P-gp and *mdr1* gene expression could constitute a mechanism for multi-drug resistance reversal not interfering with drug binding to P-gp[12,27]. Additional mechanism of in vitro multi-drug resistance distinct from P-gp expression has also been reported. Altered activity and expression of the enzyme topoisomerase II has been shown to produce resistance to the anthracyclines and the epipodophyllotoxins[15]. Interestingly, however, unlike the effect of treatment with hydroxychloroquine, chloroquine and primaquine were not found to decrease the cell viability on glioma cell lines in our study even though they were classified into the same anti-malaria drugs. It remains unclear by which reason chloroquine and primaquine don't induce a significant decrease in glioma cell viability.

Another finding of the present experiment is that hydroxychloroquine induces a marked decrease in glioma cell viability at its lowest level of each anti-cancer drug on both U87MG and T98G cell lines in a dose-dependent manner. That is, we can draw the conclusion that the treatment with hydroxychloroquine prior to anti-cancer chemotherapy maximizes its tumoricidal effect on malignant glioma cells and reverse chemotherapy resistance with acceptable drug toxicity.

However, this experiment is limited by the following fact;

1) both pharmacokinetic and hemodynamic perspectives between anti-cancer drug and anti-malaria drugs are not taken into account. 2) Even if malignant gliomas express *mdr1*, multiple or redundant mechanisms of resistance may be present in tumor cells. 3) Inability to achieve adequate drug concentrations in vivo that are active in vitro. 4) The populations of cells in malignant gliomas are heterogeneous with respect to drug sensitivity and response to reversal agents of multi-drug resistance[2]. Therefore, it is important to study local delivery of agents that modulate other mechanism of drug resistance. Although controlled clinical studies will be required, our results suggest that hydroxychloroquine can be used in the chemotherapy of malignant gliomas and its potential role in malignant glioma deserves clinical investigation.

Conclusion

The findings of this study demonstrate that hydroxychloroquine induces a dramatic tumoricidal effect of chemotherapeutic agents on malignant glioma cell lines in vitro and may represent a significant novel therapeutic approach to malignant gliomas.

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