Bull Yamaguchi Med Sch 37(3-4): 131-135, 1990

Effects of Glycyrrhizin on C6 Glioma Cells, with Special Reference to Cell Proliferation and Cell-Specific Proteins

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(Received May 7, revised July 16, 1990)

Abstract The effects of glycyrrhizin (GL) on cell proliferation, cell viability, and expression of cell-associated proteins, such as glial fibrillary acidic protein (GFAP), vimentin and S-100 protein, were estimated using an ethylnitrosourea (ENU)-elicited rat glioma cell line (C6). Graded concentrations of GL, 1.0, 1.5 and 2.0 mg/ml, were added individully to C6 cultures every 24 h and examined up to 72 hour. Cell viability was determined using a propidium iodide (PI) exclusion method. Cell cycle analyses were performed on the DNA histrogram using flow cytometry. We also investigated changes in the expression of proteins by the FITC/PI double-staining technique using flow cytometry. Cell proliferation was retarded and the number of the dead cells was increased dose-dependently by GL treatment. In cells treated with 1.0 or 1.5 mg/ml GL, cell-cycle alterations indicating an increase of the G0/G1 phase and a decrease of the S and G2M phases from the first day, were similar to those in the control group. In cells treated with 2.0 mg/ml GL, the fraction of G0/G1-phase cells did not increase, probably because the excessive dose of GL had an intense cytotoxic effect, resulting in paucity of cells in culture. These results suggest that cells were eventually lost from all phases of the cell cycle, in a dose-dependent manner and that the main effect of GL on cell proliferation was direct cytotoxicity. Maximum exposure to GL caused increased expression of each cell-specific protein, especially GFAP. However, no obvious clarification of the phenotypic alteration induced by GL treatment was obtained.

Key Words: Glycyrrhizin, C6 glioma cell, Cell proliferation, Specific protein

Introduction

Glycyrrhizin (GL) has been generally used for patients with chronic hepatitis and allergic or inflammatory disease. Recently, methods for intensifying the immune response, such as inhibitory effects against viral proliferation[1,2] and stimulation of interferon production[3], have been introduced. Hattori et al. demonstrated that GL had a protective or therapeutic effect in patients with acquired immune deficiency syndrome (AIDS)[4]. However, to our knowledge, there is no precise report about the effects of GL on the proliferation activity of tumor cells. In this brief communication, the effects of GL on cell proliferation are described, based on the tests using an ethylnitrosourea (ENU)-elicited rat glioma cell line (C6). We also investigated changes in the expression of

brain−specific proteins, such as glial fibrillary acidic protein (GFAP), vimentin and S−100 protein, in order to clarify the phenotypic alteration induced by GL treatment.

Materials and methods

Cell preparations

Exponentially growing C6 glioma cells were harvested using 0.04% trypsin solution and cultured routinely for 24 hour in 10−cm−diameter plastic culture dishes (Falcon Plastic, U.S.A.) containing Dulbecco's modified Eagle medium (Nissui Seiyaku Co., Tokyo) with 10% fetal calf serum at 37 ℃ in a humidified incubator in an atmosphere of 95% air and 5% CO_2.

Treatment with GL

GL (2 mg/1ml saline) was generously provided by Minophagen Pharmaceutical Corporation (Tokyo, Japan). Twenty−four hours after subculture, a small aliquot of GL solution was added to the existing culture medium, resulting in the following concentrations of GL: 1.0, 1.5 and 2.0 mg/ml. The agent and medium were reserved every 24 hour, and the cultures examined for up to 72 hours. At 24−h intervals, the cells were peeled from the dishes using 0.04% trypsin solution, and the following experiments except for immunofluorescence staining were performed. In every experiment, untreated cells were prepared, and the following experiments were each repeated three times.

Cell growth

The cells were counted with a Coulter counter Model ZBI (Coulter Electronics INC., Florida, U. S.A.).

Cell viability

Cell viability was determined by the propidium iodide (PI, Calbiochem Co., U.S.A.) exclusion method. Briefly, after cells floating in the medium had been removed, 50 μl of cells peeled off using 0.04% trypsin solution was added to slides with 10 μl of PI. By light microscopy, cells incorporating PI were considered to be dead, and viability was calculated within 2 min after exposure of the cells to PI.

Analysis of the cell cycle using flow cytometry

Analysis of the cell cycle was performed in order to investigate whether there was acumulation of cells in a certain phase by GL treatment. Cells peeled off with 0.04% trypsin solution were suspended in 0.2% Triton X−100 (Sigma Chemical Co., U.S.A.) diluted in phosphate−buffered saline (PBS) and 0.1% RNase (Sigma Chemical Co.). After filtration through a 40 μm−nylon mesh, PI was added at a concentration of 50 μg/ml.

Immunofluorescent staining of GFAP, Vimentin and S−100 protein.

For C6 cells exposed for 24 hours to GL, GFAP, vimentin and S−100 protein were measured to investigate the effects of GL on phenotypic alteration. Exposed cells, peeled off with 0.04% trypsin solution, were fixed in 70% cold ethanol and stored in a refrigerator until analysis. After storage for several days at 4 ℃, the fixed cells were rinsed with PBS. They were then resuspended in 500 μl PBS containing 50 μl of neutral goat serum, 0.5% Tween 20 (Sigma Chemical Co.), 0.5% bovine serum albumin, and 10 μl of monoclonal antibody for 1 hour at room temperature. Antibodies against vimentin (Dako Co., Denmark), GFAP (Dako Co.) and S−100 protein (Dako Co.) were used as primary antibodies. After washing twice with PBS, the cells were incubated in PBS containing FITC−conjugated goat anti−mouse lgG antibody (diluted 1 : 25, Dako Co.) for vimentin and GFAP, and anti−rabbit lgG antibody (diluted 1 : 25, Dako Co.) for S−100 protein. As a control, an aliquot of the cells was exposed only to the fluorescent second antibody. After resuspension with 0.1% RNase (Sigma Chemical Co.) at room temperature for 10 min, DNA staining was perfomed with PI.

Flow−cytometric analysis was performed with a FACS analyzer (Becton Dickinson Co., U. S.A.). Red fluorescence from PI was detected via a 580−nm band−pass filter and recorded as a measure of DNA content. Green fluorescence from FITC was detected via a 530−nm band−pass filter. For estimation of the percentage of FITC−labeled cells, the threshold was set using control cells.

Results

Although minor variations between each experiment occurred, the three experimental runs gave reproducible results.

Cell growth

The cells grew exponentially with a dou-

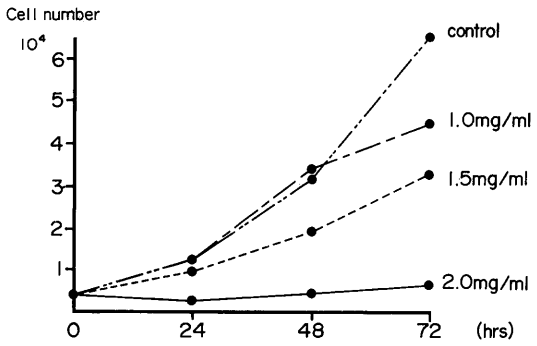


Fig. 1 Correlation between period of GL treatment and relative cell number (logarithmic scale). Cell proliferation is retarded dose-dependently by treatment with GL.

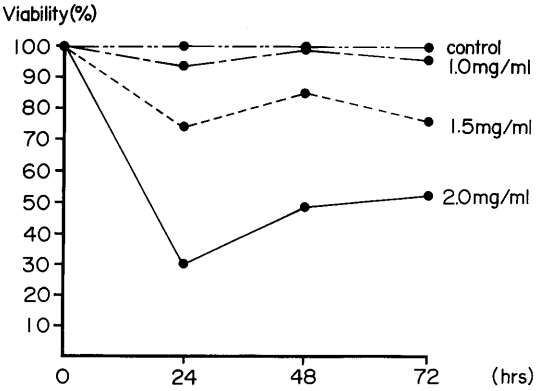


Fig. 2 Correlation between period of GL treatment and numbers of viable cells. A direct dose-dependent cytotoxic effect is shown.

bling time of approximately 15 hours under the standard culture conditions (Fig. 1). Cell proliferation was retarded by treatment with GL, dose-dependently. Cell proliferation was completely inhibited at a concentration of 2.0 mg/ml GL. Perturbation of cell growth was apparent as early as 24 hours after the first exposure.

Cell viability

Viable cells tended to be lower percentage at higher GL concentrations (Fig. 2). A direct dose-dependent cytotoxic effect was observed.

Analysis of the cell cycle using flow cytometry

On the DNA histogram, exposure of the C6 cells to this agent increased the G0/G1-phase fraction and decreased the G2M-and S-phase fractions, in the 1.0 mg/ml-and 1.5 mg/ml-treated groups, as well as in control group (Fig. 3). In the 2.0 mg/ml-treated group, however, there was no change in the percentage of each phase from the first untreated day.

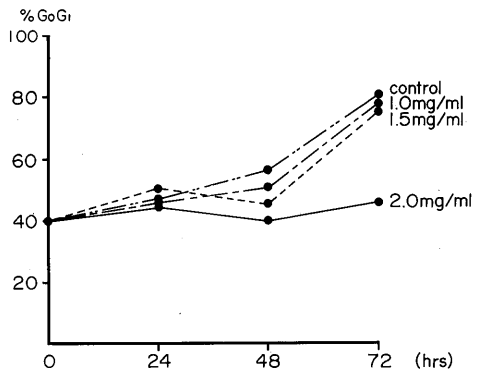
Immunofluorescence staining for GFAP, vimentin and S-100 protein (Fig. 4)

The size of the positive fraction for each the three markers was not significantly affected by exposure to any concentration of GL. Each average fluorescence intensity of FITC, representing the amounts of these cellular markers, did not increase except in the 2.0 mg/ml group, which showed slight increases in S-100 and vimentin expression and a significant increase in GFAP.

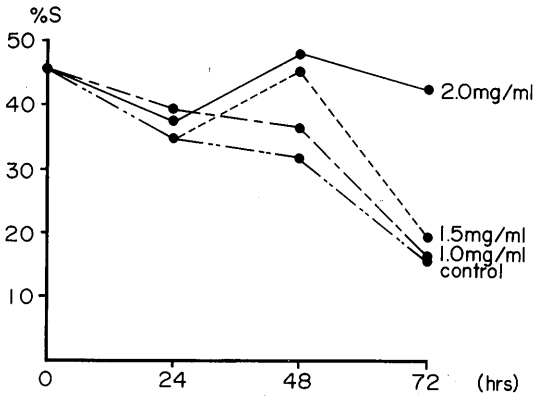
Disucussion

On the basis of the data on cell viability, together with the growth curve and DNA histogram, C6 cells were eventually lost from all phases of the cell cycle, in a dose-dependent manner. It was also apparent that the main effect of GL upon cell proliferation was direct cytotoxicity. Sasaki et al. demonstrated that evident change in growth rate was seen at a concentration of 1 mg/ml, and no growth inhibition was evident in a medium containing GL of 0.2 mg/ml using hepatoma cell line, HuH-7[5]. His results indicate that GL does not affect C6 cells exclusively.

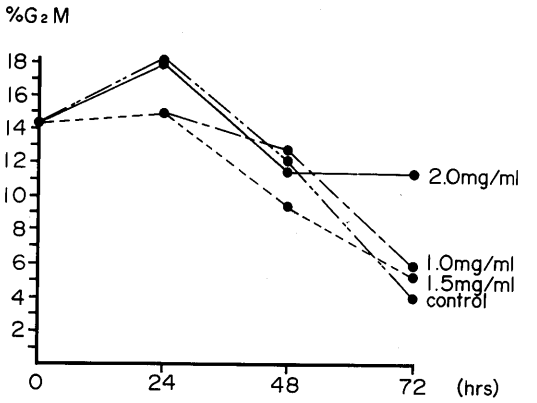
The increase of G0/G1-phase cells and the decrease of S- and G2M-phase cells according to the duration of culture in all groups except the 2.0 mg/ml GL group, seemed to be due to the fact that the cells had attained confluence. The cells in the control group became confluent earlier than the cells treated with GL. In the cells treated with 2.0 mg/ml GL, the fraction of GO/G1-phase cells did not increase, probably because the excessive dose of GL had an intense cytotoxic effect and produced a paucity of cells in culture.



(a)

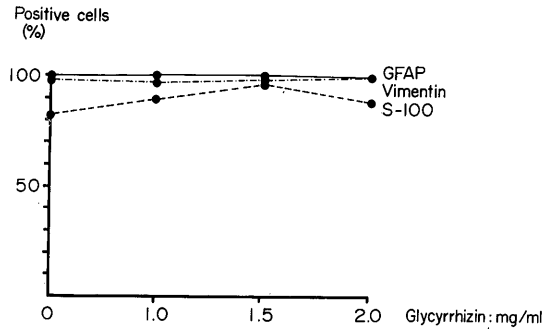


(b)

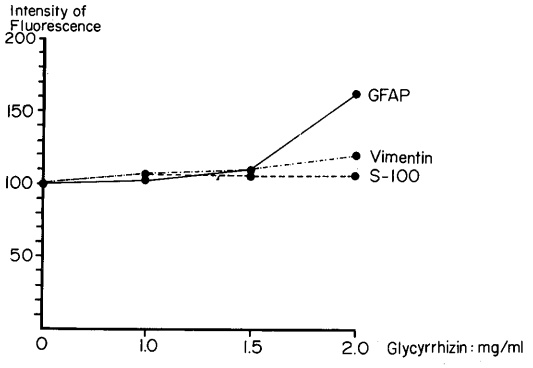


(c)

Fig. 3 Correlation of period of GL treatment and each cell cycle phase. (a) : G0/G1 phase, (b) : S phase, (c) : G2M and S phase.



(a)



(b)

Fig. 4 (a) : Correlation between concentration and no. of cells positive for each protein. (b) : Correlation between GL concentration and mean intensity of fluorescence of each protein.

The size of the positive fraction for each of the three markers was not affected by exposure to any concentration of GL. As fluorescence intensity reflects the quantity of the antigen, it is possible that a high dose of GL induces oncobiological alterations and enhances specific-protein synthesis in C6 cells. However, as the increases in these markers were not dose-depentent, it seems undeniable that the qualitative alteration in the cell membrane by excessive exposure to GL increased the permeation of antibodies through the membrane. Therefore, in this series, no conclusive answer was obtained concerning alterations of specific protein expression by exposure to GL.

GL has been demonstrated to promote the

induction of interferon *in vivo*[6], and is therefore a promising agent for use as a biological response modifier (BRM). Moreover, GL is known to inhibit the promotion of oncogenesis[7,8] and our present results may also support the potential of GL as a BRM. However, clinical use of GL may be limited, since a concentration of 1.5−2.0 mg/ml cannot be easily maintained, and GL is reported to show poor permeation through the blood−brain barrier[4]. Although the effectiveness of GL on tumor growth inhibition should be estimated after analyzing the mechanisms of its cell−growth inhibition and pharmacokinetics *in vivo*, no precise report has yet appeared. Confirmation of the practical effectiveness of GL requires investigations of its pharmacokinetics, for example, differences in its concentration between blood and brain. In addition, any specificity of its toxicity, i.e. whether GL can have a role in the treatment of brain tumors, must be examined using tumor and normal cells, possibly by treating tumor−bearing animals with GL.

Acknowledgement

We are grateful to Minophagen Pharmaceutical Corporation (Tokyo, Japan) for generously providing GL.

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