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Enhancing Effect of Patented Whey Protein Isolate (Immunocal) on Cytotoxicity of an Anticancer Drug

Wayne Y. Tsai, Wen-Huei Chang, Ching-Hsein Chen, and Fung-Jou Lu

Abstract: To determine the enhancing effect of a whey protein isolate on the cytotoxicity of a potential anticancer drug, baicalein, the human hepatoma cell line Hep G2 was assigned to grow in different media for four days, and cell growth and apoptosis were investigated. The control group was grown in normal medium; the other three groups were grown in whey protein isolate (Immunocal) medium, baicalein medium, and a combination of Immunocal and baicalein. As indicated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, survival rate was significantly lower in cells grown in baicalein + Immunocal than in cells grown in baicalein alone. In contrast, there was no significant difference in survival rate of the cells grown in Immunocal. In the investigation of apoptosis, cells grown in baicalein + Immunocal showed a higher phosphatidylserine exposure, lower mitochondrial transmembrane potential, and nearly 13 times more cells undergoing apoptosis than cells grown in baicalein alone. We also demonstrated that Immunocal reduced glutathione (GSH) in Hep G2 cells by 20–40% and regulated the elevation of GSH, which was in response to baicalein. In conclusion, Immunocal seemed to enhance the cytotoxicity of baicalein by inducing more apoptosis; this increase in apoptotic cells may be associated with the depletion of GSH in Hep G2 cells. This is the first study to demonstrate, in vitro, that Immunocal may function as an adjuvant in cancer treatments.

Introduction

The tripeptide glutathione (GSH) is a low-molecular-weight thiol reductant present in most cells. It assumes a pivotal role in numerous cell functions, such as protection of cells from toxic oxygen species and detoxification of various xenobiotics (1–4). It has been demonstrated that tumor cells contain severalfold higher GSH than normal tissue cells (5), and this appears to be at least one of the mechanisms of acquired drug resistance to chemotherapy (6,7). Recently, GSH concentration in tumor cells has been suggested as a useful predictor of resistance or sensitivity to anticancer treatments (8–10). There is an increasing number of studies

done on selectively modulating intracellular GSH to sensitize cancer cells to anticancer drugs or to reduce the problem of resistance (5,11,12).

Aside from the role of GSH in the defense mechanism, programmed cell death or apoptosis is also closely correlated to this tripeptide (13). Characteristics of cells undergoing apoptosis include physiological changes, such as pyknosis and apoptotic bodies, biochemical changes, such as activation of caspases, DNA fragmentation, phosphatidylserine (PS) exposure, and mitochondrial transmembrane potential alterations. These changes are considered as markers to identify apoptotic cells. Apoptosis was reported when GSH levels were reduced or when GSH was actively extruded outside the cells (14). Bcl-2 activation was associated with the redistribution of GSH to the nucleus (15), and cells resistant to apoptosis induction were reversed when GSH was reduced (16). Furthermore, one of the causes of apoptosis, such as an increase in generation of reactive oxygen species, was shown to be accompanied by depletion of GSH (2).

Two major agents were involved in this experiment: a potential anticancer drug, baicalein, and a patent-protected whey protein isolate, Immunocal. Baicalein is one kind of flavonoid extracted from Labiatae *Scutellaria revularis*. Baicalein has been widely used in traditional Chinese medicine for many years; it is believed to have antitumor activity. Recently, a series of scientific studies gave us a closer look at this natural plant component, and through these studies, its impacts on tumor cells were confirmed. The studies demonstrated that baicalein was able to inhibit the growth rate of human hepatoma (PLC/PRF-/5), human liver (Chang liver cells), and human pancreatic cancer cell lines (17). Baicalein was also shown to inhibit T lymphoid leukemia cell proliferation through reduction of protein tyrosine kinase activity and protein kinase C activity induced by phorbol-12-myristate-13-acetate (18,19). Furthermore, it was reported that baicalein inhibited a hepatocellular carcinoma cell line (HuH-7) by interfering with DNA synthesis (20). In 1999, baicalein was demonstrated to be a potential cancer-chemopreventive agent against tumor promotion (21). Our present findings also agreed with the idea that baicalein was able to retard

cell growth at lower doses and further induce apoptosis at higher doses in human Hep G2 cells; these processes were accompanied by reactive oxygen species generation (personal communication).

The other major agent in the experiment, Immunocal, has been reported to selectively modulate GSH levels in cancer vs. normal cells (11,22–27). This whey protein concentrate is prepared in a special fashion to preserve the native forms of the cysteine-rich proteins in whey (serum albumin, lactoferrin, and α -lactalbumin) and functions as a cysteine donor system in cells. As demonstrated in some studies, when Immunocal was provided, GSH levels in lymphocytes, some human organs (25,27), normal peripheral blood mononuclear cells (11), and spleens of unimmunized mice (26) often increased. In contrast, when Immunocal was applied to tumor cells (MATB and Jurkat T cell lines), GSH levels seemed to decline (11,22) and initiation of carcinogenesis (28) and tumor progression (29) seemed to be inhibited. It was believed that the results were associated with a feedback inhibition of GSH synthesis in tumor cells.

One of the applications of GSH modulation in a clinical trial may be adjuvant chemotherapy. The function of an adjuvant is to reduce the side effects or the dosage during the cancer treatments while sustaining or promoting the anti-cancer effect. In this study, we intended to investigate the enhancement effect of Immunocal on the cytotoxicity of baicalein. We assigned the human hepatoma cell line Hep G2 to grow in different media: baicalein, Immunocal, or baicalein + Immunocal. After determining that Immunocal alone did not significantly affect cell growth and that the survival rate with baicalein + Immunocal was significantly lower than with baicalein alone, we continued with a series of investigations. Surprisingly, according to our data, the apoptosis induced by baicalein was significantly enhanced in the presence of Immunocal. This enhanced cytotoxicity provided direct evidence that Immunocal could perform as an adjuvant in cancer treatments.

Materials and Methods

Chemicals

Baicalein was purchased from Aldrich (St. Louis, MO). Dimethylsulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany). Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium dodecyl sulfate, Dulbecco's modified Eagle's medium (DMEM), streptomycin, penicillin, amphotericin B, and trypsin-EDTA were purchased from Sigma Chemical (St. Louis, MO). Fetal bovine serum was obtained from GIBCO BRL (Gaithersburg, MD). Rhodamine 123 was purchased from Calbiochem (La Jolla, CA), annexin-V-Fluorescein diacetate (CMFDA) from Molecular Probes (Eugene, OR).

Whey Protein Isolate

Immunocal was prepared according to a patented procedure and supplied as a powder by Immunotec Research (Montréal, PQ, Canada). It was pathogen free and had the following characteristics: 88–92% pure whey proteins, <0.5% fat, <3% minerals, <1.5% lactose, and <5% moisture, with a solubility index of 99% at pH 4.6. A special technique was used to preserve, in undenatured form, the cysteine with thermolabile proteins.

Cell Line

Hep G2 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in preincubation medium consisting of DMEM supplemented with 10% fetal bovine serum, 100 μ g/ml streptomycin, and 250 μ g/ml amphotericin B at 37°C in a 5% CO₂ humidified atmosphere. Cells were routinely subcultured once a week.

Treatment of Cells With Immunocal, Baicalein, or Immunocal + Baicalein

Immunocal solution was freshly prepared before each experiment. The powder was resuspended in DMEM and centrifuged at 12,000 g for 10 minutes at room temperature. The supernatant was filtered on a 0.2- μ m filter. Concentrations between 0.01 μ g/ml and 1 mg/ml were used in the experiment. The stock solution of baicalein (100 mM in DMSO) was prepared in DMEM before each experiment, with a final concentration of DMSO of \leq 0.1%. For the combined treatment, final concentrations of 25 and 50 μ M baicalein were prepared with the presence of 1 mg/ml Immunocal.

Cell Viability by MTT Assay and Trypan Blue Exclusion

Cells were seeded in 12-well plates with a density of 1×10^5 /well 12 hours before treatments. Cells assigned to grow in baicalein medium were incubated in 25, 50, 75, and 100 μ M baicalein. Cells grown in Immunocal medium were incubated in 0.01, 0.1, 1, 10, 100, and 1,000 μ g/ml Immunocal. For the combined treatments, baicalein was added to 1 mg/ml of Immunocal medium and reached final concentrations of 25, 50, 75, or 100 μ M. To determine cell viability after incubation for 48, 72, and 96 hours, a modified MTT assay as described by Carmichael and associates (30) was used. The media were replaced with MTT solution (0.5 mg/ml) for four hours, and 10% sodium dodecyl sulfate was added for 12 hours to thoroughly dissolve the dark blue crystals. Cell viability was determined by absorbance at 570 nm, measured by an Elx 800 reader (Bio-Tek Instruments). Cell number was also determined at indicated times on the basis of trypan blue exclusion. Trypsin was added to the control cells and the treated cells. After the cells were detached from the plate, they were resuspended in culture media. Equal volumes of each cell suspension and trypan blue solution [0.2% in phosphate-buffered saline (PBS)] were mixed and

used for cell counting by hemocytometer. The blue-stained cells were counted as nonviable cells and the unstained cells as viable cells.

Flow Cytometry

Flow cytometry analysis was done by Becton-Dickinson FACS-Calibur flow cytometer. Hep G2 cells were seeded in 60-mm dishes (5×10^5 cells/dish) 12 hours before experiments. Cells were then exposed to medium containing Immunocal (1 mg/ml), baicalein (50 μ M), or the combination of baicalein (50 μ M) and Immunocal (1 mg/ml) for different time courses. Subsequently, several labeling dyes such as annexin V-fluorescein 5-isothiocyanate (FITC), rhodamine 123, and CMFDA were used to investigate the events involved in apoptosis.

Detection of Apoptosis by TdT-Mediated dUTP-Biotin Nick End Labeling

An improved version of the assay described by Separovic and co-workers (31) was used. After 72 hours of incubation, cells were trypsinized and washed with PBS containing 0.2% bovine serum albumin (BSA). After they were fixed in 4% paraformaldehyde on ice for 30 minutes, cells were permeated by 70% ethanol at -20°C for another 30 minutes and then washed with PBS (0.2% BSA). Then cell pellets of $1-2 \times 10^6$ cells were resuspended in 30 μ l of reacting agent from the Mebstain apoptosis kit (Immunotech, France) and incubated for one hour at 37°C . After one hour of incubation, cells were washed with 1 ml of PBS (0.2% BSA), resuspended in 4 μ g/ml of PI, and subjected to flow cytometry analysis.

Assessment of Mitochondrial Transmembrane Potential

After 12, 24, and 48 hours of incubation in different treatment media, cells were harvested by trypsinization, washed twice with PBS, and stained with rhodamine 123 (5 μ M) in the incubator for 30 minutes (32). After they were washed with PBS, cells were resuspended in 4 μ g/ml of PI solution. PI was used to exclude the cells with disrupted plasma membrane. Mitochondrial transmembrane potential was detected by the fluorescence of rhodamine 123.

PS Exposure

PS exposure was measured by using a fluorescence conjugate of the natural ligand, annexin V (33). After 24, 48, and 72 hours of exposure in different treatment media, cells were labeled with a final concentration of 5 μ l/ml of annexin V-FITC for 15 minutes in the incubator. Cell pellets were washed twice with PBS, transferred to 1 ml of PBS containing 4 μ g/ml of PI, and then subjected to flow cytometry analysis.

GSH Determination

For thiol level analysis, the cells were loaded using the method previously described (34). The advantage of this method was that only viable cells could react with CMFDA,

and our main interest was the GSH contents in surviving cells. Briefly, the cells treated with baicalein, Immunocal, or baicalein + Immunocal were trypsinized, incubated in PBS containing 25 μ M CMFDA at 37°C for 10 minutes, and subjected to flow cytometry analysis.

Results

Enhancing Effect of Immunocal on Cytotoxicity of Baicalein

The enhancing effect of Immunocal was evaluated by comparing survival rates of cells treated with baicalein alone and cells treated with baicalein + Immunocal. MTT assay was performed to reflect the survival rates. As shown in Figure 1A, Immunocal (0.01–1,000 μ g/ml for 4 days) alone had no significant effects on Hep G2 cell viability. As shown in Figure 1, B–D, viability was significantly reduced time and dose dependently when cells were treated with 25, 50, 75, and 100 μ M baicalein. However, when the same doses of baicalein were applied to the cells in the presence of 1 mg/ml of Immunocal, a further increase of mortality was demonstrated. The enhanced cytotoxicity was clearly observed with combined treatment with baicalein (25 and 50 μ M) and Immunocal (1 mg/ml). At higher concentrations of baicalein (>50 μ M), the cell survival rates were too low and the enhancement was difficult to see. To clarify whether the decrease of cell viability was due to growth inhibition or cell death, the actual number of cells in each group was further calculated. In Figure 2, control and Immunocal-treated groups showed a sigmoidal increase in cell numbers; in the cases of treatment with baicalein and baicalein + Immunocal, the increase in cell numbers was slowed, indicating an imbalance between cell proliferation and cell death. In both groups, baicalein (at 50 μ M) alone and baicalein (at 50 μ M) + Immunocal, the cell numbers were significantly lowered at all time intervals measured. We thus focused on these doses for our subsequent experiments. In Figure 3, the cell cycle of each group at different time courses was analyzed. Compared with the control group, treatment with baicalein and baicalein + Immunocal showed a significant increase in the G_0/G_1 phase and a decrease in the S phase. However, except for 72 hours, the percentage of the G_0/G_1 or the S phase was not significantly different between the group treated with baicalein alone and the group treated with baicalein + Immunocal, indicating that some mechanisms other than growth retardation may contribute to the enhanced cytotoxic effect of baicalein by Immunocal.

Apoptosis of Hep G2 Enhanced by Immunocal

We further investigated the pathway of cell death. As shown in Figure 4, DNA fragmentation, a characteristic event in apoptosis, was indicated by TdT-mediated dUTP-biotin nick end labeling assay. The gated region reflected the percentage of total cells that underwent apoptosis. At 72

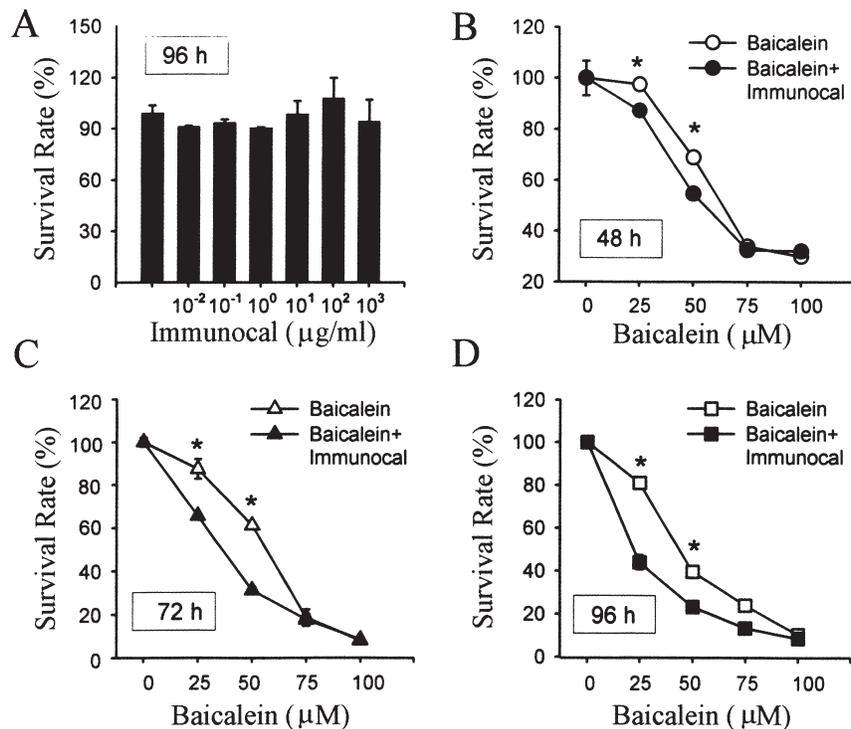


Figure 1. Survival rates of Hep G2 cells under different treatments. A: cells treated with Immunocal (0.01–1,000 µg/ml) for 96 h. B, C, and D: cells treated with baicalein (25, 50, 75, and 100 µM) and/or 1 mg/ml Immunocal for 48, 72, and 96 h, respectively. Viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Dimethylsulfoxide served as vehicle of baicalein. All data were compared with control groups. Each plot represents mean \pm SE ($n = 4$). *, Significantly different from baicalein + Immunocal, $p < 0.05$.

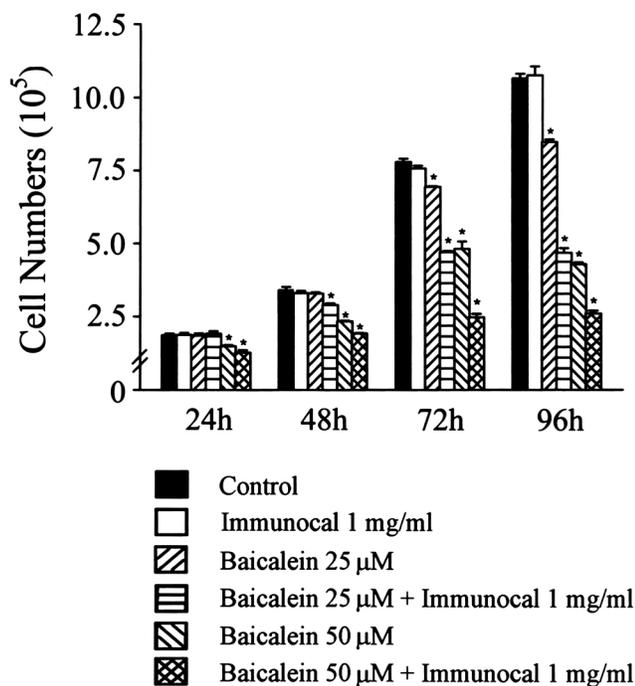


Figure 2. Alterations of cell numbers under different treatments. Cells (1×10^5 /well) were treated with indicated doses of baicalein or 0.01% dimethylsulfoxide in presence or absence of Immunocal. At end of experiments, cell numbers of each group were calculated by trypan blue exclusion assay. Values are means \pm SE ($n = 4$). *, Significantly different from control, $p < 0.05$.

hours, 0.69% and 0.65% of the cells were in the gated region in the control and Immunocal groups, respectively. In the baicalein (50 µM) group, there was a significant increase: 2.37% of the cells underwent apoptosis. In the group treated with baicalein (50 µM) + Immunocal (1 mg/ml), 30.97% of the cells were detected. Nearly 45 times more cells were detected in the group treated with baicalein + Immunocal than in the control group, and 13 times more cells were detected than in the group treated with baicalein alone.

Loss of Mitochondrial Transmembrane Potential

The alternation of mitochondrial function has been proposed to correlate to cell damage (35). Disruption of mitochondrial function, such as loss of transmembrane potential, at an early and irreversible stage occurred in apoptosis, as demonstrated by the reduction of rhodamine 123 fluorescence. In Figure 5, we demonstrated that, after 12 hours of incubation in different media, rhodamine 123 fluorescence in the group treated with 50 µM baicalein was 64.7% of that in the control group and in the group treated with Immunocal (1 mg/ml) + baicalein was further reduced to 48.2% of the control group. This effect by Immunocal was statistically significant and continued for 48 hours.

Elevation of PS Exposure

The loss of mitochondrial transmembrane potential is usually accompanied by a subsequent alternation of plasma

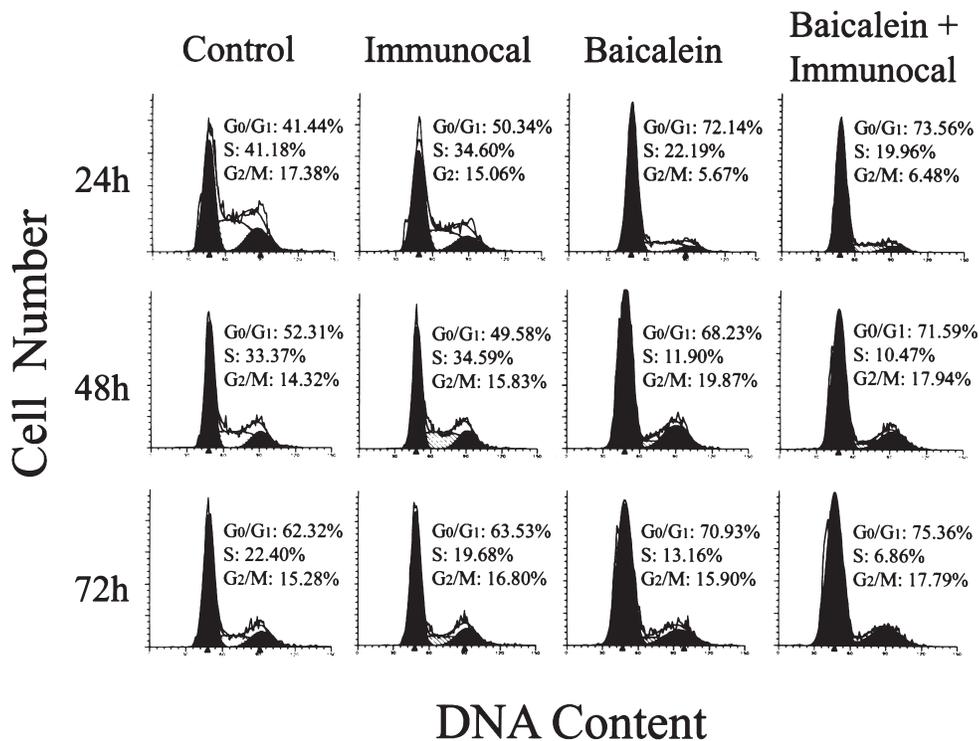


Figure 3. Effects of different treatments on cell cycle of Hep G2 cells. Cells were treated with Immunocal (1 mg/ml), baicalein (50 μ M), or baicalein + Immunocal for indicated time periods. Cells were then harvested, stained with propidium iodide, and analyzed by flow cytometry. Data are from 1 of 3 similar experiments and are presented as percentage of total cells in G₀/G₁, S, and G₂/M phases of cell cycle.

membrane. By using annexin V-FITC, cells with the externalization of inner membrane phospholipid, PS, were identified. Because the translocation of PS could occur during necrosis, PI was used in conjunction with annexin V-FITC to exclude the necrotic cells. PI-negative cells were gated and compared as shown in Table 1. After 48 hours, annexin

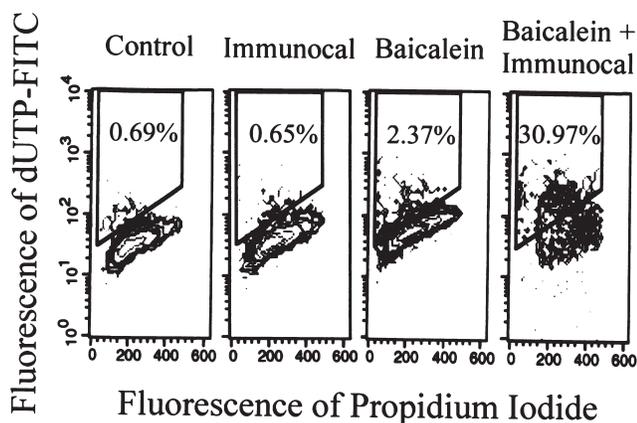


Figure 4. Apoptosis of Hep G2 cells enhanced by Immunocal. Apoptosis was evaluated by TdT-mediated dUTP-biotin nick end labeling assay. Cells in gated region were labeled with dUTP-fluorescein 5-isothiocyanate by TdT enzyme and identified as apoptotic cells. At 72 h, fluorescence emitted from dUTP-fluorescein 5-isothiocyanate of 4 groups (control, Immunocal, 50 μ M baicalein, and 50 μ M baicalein + 1 mg/ml Immunocal) was analyzed by flow cytometry. Results were from 1 experiment that was representative of 3 similar experiments.

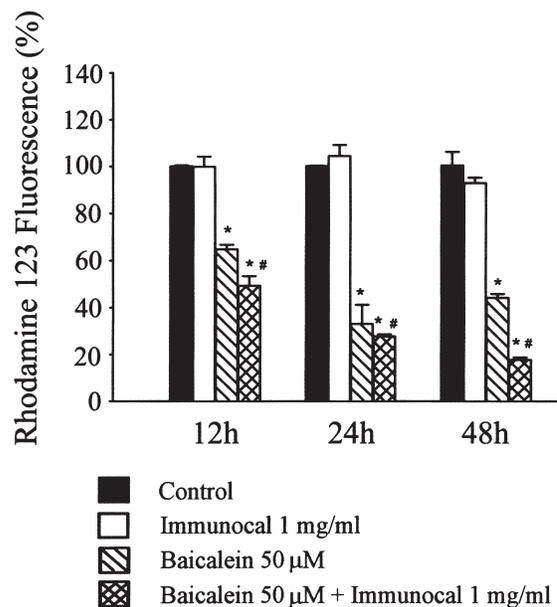


Figure 5. Changes in mitochondrial transmembrane potential. Cells under different treatments were stained with rhodamine 123 and analyzed by flow cytometry. Baicalein significantly lowered transmembrane potential as early as 12 h. With Immunocal, reduction was enhanced. Values are means \pm SE ($n = 4$). *, Significantly different from control; #, significantly different from baicalein.

Table 1. Alteration of Plasma Membrane Under Different Treatments^a

Treatment	24 h	48 h	72 h
Control	100 ± 0.68	100 ± 0.88	100 ± 0.195
Baicalein (50 μM)	75.85 ± 0.62	126.38 ± 1.14	168.06 ± 4.88
Baicalein (50 μM) + Immunocal (1 mg/ml)	105.61 ± 0.65	653.93 ± 6.80	253.69 ± 0.86

a: Values are means ± SE; n = 3. Annexin V fluorescence of cells treated with different media was compared. Fluorescence of control groups was normalized to 100%; fluorescence of groups treated with baicalein and baicalein + Immunocal is shown as percentage relative to control. At 48 h, phosphatidylserine exposure in baicalein + Immunocal group was nearly 5 times more than in baicalein group. At 72 h, phosphatidylserine was ~1.5 times greater in cells treated with baicalein + Immunocal than in cells treated with baicalein alone.

V-FITC labeling increased in the baicalein group to 126.38% of control, but in the presence of Immunocal more than a sixfold increase was observed. After 72 hours, cells treated with baicalein + Immunocal still showed a higher percentage of fluorescence (253.69%) than cells in the single-treatment groups (168.06%).

Intracellular GSH Modulation by Immunocal

First, we tested the GSH levels of Hep G2 cells when Immunocal was applied alone, in different doses (0.01 μg/ml to 1 mg/ml) and over different time courses (12–72 h). We did not observe an increase in GSH levels, but we did observe a reduction, to 80% of the control groups, at 500 μg/ml and 1 mg/ml at 12, 24, 48, and 72 hours (data not shown).

We then investigated the correlation between GSH levels and apoptosis in different treatments. As shown in Figure 6, GSH levels in cells treated with Immunocal were ~20–40% below GSH levels in the control group. In the group treated with baicalein, >50% of GSH was exhausted at 24 hours, but

starting from 48 hours, GSH levels rose significantly. At 72 hours, the concentrations reached ~180% compared with control. Compared with the baicalein case, the elevation of GSH concentrations was effectively reduced in the groups treated with baicalein + Immunocal.

Discussion

In this study, we were able to demonstrate that the cytotoxicity of baicalein to Hep G2 cells was significantly enhanced by Immunocal. More cells in the group treated with baicalein + Immunocal underwent the process of apoptosis than in the group treated with baicalein alone. This enhancement was supported by higher PS exposure, lower mitochondrial transmembrane potential, and regulation of GSH levels in the cells. These findings imply the potential use of Immunocal in adjuvant chemotherapy.

Killing tumor cells without endangering normal tissue surrounding them has always been an obstacle in cancer treatments. Much research and many studies have focused on selectively manipulating cellular GSH levels to overcome this problem (36). Traditionally, GSH depletion can be effectively achieved by buthionine sulfoximine (BSO), which inhibits the enzyme γ-glutamylcysteine synthase in the GSH synthesis cycle (37). On the other hand, few agents are available to promote intracellular GSH concentrations, agents such as N-acetylcysteine (NAC), oxothiazolidine carboxylate (OTZ), GSH esters, and bioactive whey proteins (38). NAC carries certain toxicity itself and is known to have a short half-life; as a consequence, rapid elevations are followed by rapid declines that can decrease GSH even below baseline levels (39–42). OTZ, when enzymatically cleaved by 5-oxoprolinase, yields high concentrations of the GSH precursor cysteine, which stimulates GSH synthesis. However, the required enzyme 5-oxoprolinase is not present in all types of cells; thus the value of OTZ is limited (43). The synthetic compounds, GSH esters, are effective GSH delivery systems, but they have the disadvantage of being metabolized into alcohol (44,45). Alcohol (ethanol) can potentially deplete GSH (46). Whey, a good choice for raising GSH levels, was treated for a long time as an insignificant by-product of the dairy industry but is now the subject of a new surge of interest. Whey contains naturally the proteins that are rich in cysteines; cysteine is the crucial precursor

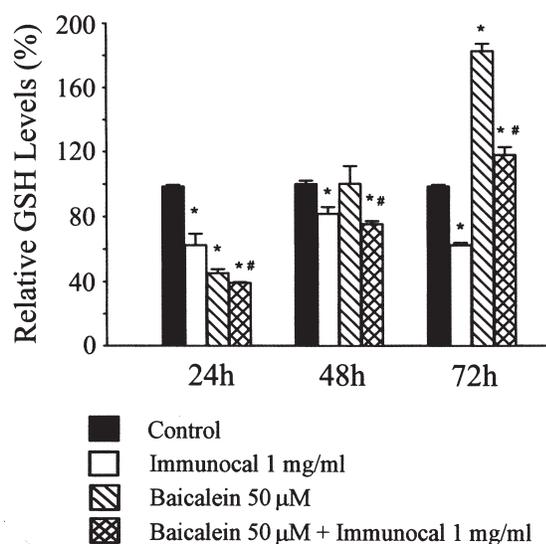


Figure 6. Intracellular glutathione (GSH) levels under different treatments. Hep G2 cells in each treatment were stained with 5-chloromethylfluorescein diacetate and analyzed by flow cytometry. Values are means ± SE (n = 4). *, Significantly different from control; #, significantly different from baicalein.

sor for GSH synthesis. However, many variables, such as concentration of total protein, types of proteins, degree of denaturation (breakdown), fat content, lactose content, bioavailability, biological activity, and contaminants (47), may play a role in the effects of whey products.

In our experiment, Immunocal, a whey protein concentrate prepared by a special technique, was used. It has been reported to contain bioactive proteins. Bioactive proteins, in the case of whey proteins, refer to the proteins with biological activities that remain in their undenatured forms when passing through the stomach and function in delivery of cysteines at the cellular level and, eventually, augment GSH levels inside the cells (23). Immunocal has also been demonstrated to selectively manipulate GSH levels in normal vs. cancer cells (11,22–27). Yet, there has been no evidence that GSH is able to freely pass through cell membranes; thus Immunocal must modulate GSH levels by stimulating cells to synthesize GSH intracellularly. Immunocal is rich in proteins such as serum albumin, lactoferrin, and α -lactalbumin; these active proteins are effective cystine donors. There are 17 cystine residues per 66,000-mol wt molecule of serum albumin (48), 17 cystine residues per 77,000-mol wt molecule of lactoferrin (49), and 4 cystine residues per 14,000-mol wt molecule of α -lactalbumin (48). According to the study of Baruchel and Viau in 1996 (11), Immunocal seemed to be able to selectively modulate cellular GSH in normal cells and rat mammary carcinoma. In their experimental model, intracellular GSH concentrations in tumor cells were elevated when a small dose of Immunocal was applied; GSH concentrations were reduced when a large dose was applied (11). These findings are interesting, but the mechanisms remain to be discovered. However, in our experiment, the elevation of GSH concentrations did not appear in different time courses or different doses (data not shown), but we did observe the reduction of GSH concentrations when Immunocal was applied. We speculated that this reduction may be correlated with the increasing number of apoptotic cells in groups treated with Immunocal + baicalein.

In this experiment, we initially observed that the cell survival rates were reduced by baicalein, and they were further reduced in the presence of Immunocal (Figures 1 and 2). To investigate the causes of this enhancement, more investigations were subsequently done. As shown in Figure 3, we found that treatment with baicalein alone and with baicalein + Immunocal resulted in arrest of the same percentage of the total cells in the G₁ phase. In Figure 4, many more apoptotic cells were found in the group treated with baicalein + Immunocal than in the group treated with baicalein alone. We correlated this enhancement of cytotoxicity with the increasing cells in apoptosis. To investigate the possible mechanism that may be in association with apoptosis, we evaluated the mitochondrial transmembrane potential and PS exposure. A considerable amount of research has demonstrated the correlation between loss of mitochondrial transmembrane potential and apoptosis (50). As seen in Figure 5, when cells were treated with baicalein alone, rhodamine 123 fluorescence decreased. Treatment with baicalein + Immu-

nocal decreased rhodamine 123 fluorescence further. The loss of mitochondrial transmembrane potential is usually accompanied by a subsequent alternation of plasma membrane. We examined PS exposure and found that a higher percentage of fluorescence was shown in cells treated with baicalein + Immunocal than that in cells treated with baicalein alone (Table 1). At 24 hours, there was a decrease of PS exposure in the groups treated with baicalein. According to previous studies, flavonoid could protect the cell membrane in some cases (51). Finally, because Immunocal was believed to modulate GSH levels, we measured the intracellular GSH content and found that Immunocal alone could reduce the GSH synthesis. In baicalein-treated groups, GSH levels were initially lower than control; this might be the result of active detoxification of the cells. At 72 hours, the cells that survived baicalein treatment showed elevated GSH contents that were nearly 180% of control. This augmentation of GSH levels may play a major role in keeping the cells alive in this case; we speculated that this elevation may be in association with drug resistance. Furthermore, the cells from the group treated with baicalein + Immunocal did not show the highly elevated GSH levels as seen in the group treated with baicalein alone, the GSH concentrations were regulated by Immunocal, and we correlated this depletion of GSH with the increase of apoptosis.

To a physician, as well as to a patient who is receiving chemo- or radiotherapy, an optimal condition would be reduction of GSH concentrations in tumor cells along with augmentation of GSH concentrations in normal cells. In this scenario, a lower-dose treatment would be required to kill tumor cells, and side effects on the normal tissue would be minimized. Indeed, more laboratory studies and clinical data suggested that Immunocal could produce this result. In conclusion, the whey protein isolate Immunocal was able to enhance the cytotoxicity of the potential anticancer drug baicalein on Hep G2 cells. We correlated this enhancement with the modulation of GSH by Immunocal. In future application, Immunocal may provide some benefit in cancer treatments.

Acknowledgments and Notes

The authors acknowledge the technical support and advice of G Bounous and the editing support of Bonny Yee. Address correspondence to Prof. Fung-Jou Lu, Dept. of Biochemistry, College of Medicine, National Taiwan University, No. 1, Sec 1, Jen-Ai Rd., Taipei, Taiwan, ROC. Phone: 886-2-2312-3456 (ext. 8208). FAX: 886-2-2321-4559. E-mail: fujulu@ha.mc.ntu.edu.tw.

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