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Combination of aspartic acid and glutamic acid inhibits tumor cell proliferation

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ABSTRACT

Placental extract contains several biologically active compounds, and pharmacological induction of placental extract has therapeutic effects, such as improving liver function in patients with hepatitis or cirrhosis. Here, we searched for novel molecules with an anti-tumor activity in placental extracts. Active molecules were separated by chromatographic analysis, and their antiproliferative activities were determined by a colorimetric assay. We identified aspartic acid and glutamic acid to possess the antiproliferative activity against human hepatoma cells. Furthermore, we showed that the combination of aspartic acid and glutamic acid exhibited enhanced antiproliferative activity, and inhibited Akt phosphorylation. We also examined *in vivo* tumor inhibition activity using the rabbit VX2 liver tumor model. The treatment mixture (emulsion of the amino acids with Lipiodol) administered by hepatic artery injection inhibited tumor cell growth of the rabbit VX2 liver. These results suggest that the combination of aspartic acid and glutamic acid may be useful for induction of tumor cell death, and has the potential for clinical use as a cancer therapeutic agent.

Hepatocellular carcinoma (HCC) is the sixth most common and aggressive malignancy in the world (22). Poor prognosis in HCC is the major cause of cancer-related deaths, and each year more than 500,000 new patients are diagnosed with HCC worldwide (4). Furthermore, increasing incidence has been projected through 2020 (22). Various types of therapies are available for HCC such as transarterial therapy with or without embolization, systematic therapy, interferon, and lamivudine. Although conventional chemotherapy is well tolerated in inoperable HCC patients (27, 32), various adverse effects have been reported for interferon (8). In addition, resistance to chemotherapy due to long-term administration of anticancer drugs is widely identified in HCC patients; the response rates are approximately 20% for single agent as well as combination chemotherapy (29). In addition, the 3-year tumor recurrence rate was under 60% following hepatic resection (19) and the 5-year overall survival rate ranged between 39% and 50% (14, 23). Therefore, cure rates are not satisfactory (2).

Placental extract can be obtained by hydrolysis of placenta via both hydrochloric acid and enzymatic digestion. It has several biological activities, such as stimulation of liver regeneration (28), anti-oxidation (26), and anti-xanthine oxidase activity (30). Owing to these multiple functions, placental extract is expected to have wide applications in the healthcare field. In fact, human placental extract has been clinically administered to improve liver function in patients with hepatitis or cirrhosis in Japan for more than 50 years. Based on the cell growth-promoting activity of placental extract (1, 28), we hypothesized that placental extract may be a useful substitute for fetal bovine serum in cell culture systems. However, contrary to our expectations, placental extract inhib-

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ited growth of various cell lines such as B16 (mouse skin melanoma) and HepG2 (human hepatoma) cells, but not primary cells such as rat hepatocytes. This finding suggested that placental extract contains compounds with antiproliferative activity. Hence, we searched for novel compounds with anti-cancer activity in placental extract using cell-based assays. We used HepG2 cells for the following two reasons: placental extract has been used in clinical practice for treatment of hepatic diseases, and our pilot cell growth assay showed that placental extract inhibited the proliferation of these cells.

In the present study, we identified compounds with antiproliferative activity in placental extract using chromatographic analyses, and determined the optimal combination of these compounds for effectiveness. The antiproliferative effects of these compounds on HCC tumor cells were evaluated in HepG2 cells. To determine the signaling pathways involved in the antiproliferative activity, we examined kinase phosphorylation associated with cell growth or survival. Furthermore, we evaluated the efficiency of these compounds *in vivo* in a conventional animal tumor model.

MATERIALS AND METHODS

Chemicals. Most chemicals were purchased from Wako Chemicals (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan).

Cell line and cell culture. The placental extract was obtained from Japan Bio Products Co., Ltd. (Tokyo, Japan). HepG2 (hepatoma), Huh-7 (hepatocellular carcinoma), and HLE (hepatoma) cells were purchased from Health Science Research Resources Bank (Osaka, Japan). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in humidified air containing 5% CO_2.

Cell viability assay. The cells were plated at 1.5×10^3 cells/well in 96-well plates, and cultured for 24 h. On the following day, the test samples were added, and the cells were cultured for an additional hour. Cell viability was determined by sulforhodamine B.

Purification and identification of active components. The placental extract was separated by Tangential Flow Filtration system (Millipore, Bedford, MA, USA). The active fractions of the placental extract were subjected to ion exchange chromatography SP-sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and then applied to a high-performance liquid chromatography (HPLC) uRPC C2/C18 ST4.6/100 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA). This procedure was repeated twice. The separated active components were analyzed by postcolumn derivatization with *O*-phthaldialdehyde using HPLC.

Western blot analysis. Cells were plated at 8.0×10^5 cells/60-mm dish and allowed to grow overnight. Then cells were treated with compounds in serum containing DMEM medium for 2 h. After treatment, cells were lysed in cell lysis buffer containing protease and phosphatase inhibitors. Cell lysates (20 µg protein) were loaded onto 10% SDS-PAGE gels, electrophoresed under reducing conditions, and transferred onto PVDF membranes. Blots were probed with anti-phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204; D13.14.4E; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-p44/42 MAPK (Erk1/2; 137F5; Cell Signaling Technology, Inc.), anti-AKT (AKT; pan; C6707E; Cell Signaling Technology, Inc.) and anti-phospho-AKT (Phospho-AKT; Ser473 D9E; Cell Signaling Technology, Inc.) antibodies, and horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, Inc.). Following washes, the membranes were developed with WesternSure premium chemiluminescent substrate (LI-COR; Lincoln, NE, USA) and read on a LAS-3000 LuminoImage analyzer (Fujifilm, Tokyo, Japan).

In vivo studies using rabbit VX2 liver tumors. The aqueous mixture solution of aspartic acid (4.0 mg/mL) and glutamic acid (4.4 mg/mL) containing 1.53% Tween-20 was mixed with 5.5 volumes of Lipiodol (Terumo, Japan), and stirred with an ultrasonic stirrer. VX2 carcinoma cells were maintained as a tumor line in SLC Biotechnical Center. Adult JW/CSK male rabbits weighing 2.6–3.1 kg (SLC, Shizuoka, Japan) underwent VX2 tumor implantation to the left medial hepatic lobe. The animals were used for experiments two weeks after tumor implantation. The tumors were measured with Vernier calipers, and the tumor sizes at the time of the experiment were between 1.0 and 2.0 cm in diameter. In all experiments, anesthesia was administered by intramuscular injection of a mixture of ketamine hydrochloride (1.1 mL/kg, Daiichi-Sankyo, Japan) and xylazine hydrochloride (0.4 mL/kg, Bayel, Japan). Eighteen

VX2 tumor-bearing rabbits were randomly divided into two groups (emulsion treatment group and without emulsion group), and hepatic artery injection was performed in these animals (15). The emulsion treatment group received saline (0.1 mL/kg) or aspartic acid (0.7 or 1.4 mg/kg) and glutamic acid (0.8 or 1.5 mg/kg) in 0.01% Tween-20 mixed with Lipiodol. The without emulsion group received aspartic acid (0.7 or 1.4 mg/kg) and glutamic acid (0.8 or 1.5 mg/kg) in 0.01% Tween-20. Tumor dimensions and body weights were measured 7 days after drug administration. Tumor volumes were calculated using the equation $(l \times w^2) / 2$, where l and w refer to the larger and smaller dimensions collected at each measurement. Hepatic and renal toxicities were determined by biochemical analysis of the plasma of non-tumor bearing rabbits. Blood samples were collected before and 1, 3, 7, and 14 days after drug administration.

Ethics Statement. Animal experiments were carried out in accordance with the protocol approved by the Animal Care and Use Committee and in compliance with SLC guidelines. Animals received food and water *ad libitum*.

Statistics. The *in vitro* and *in vivo* data are presented as mean ± SD from at least three replicates and were analyzed by Dunnett's test. A p-value < 0.05 was considered significant.

RESULTS

Identification of active components in the placental extract

To determine whether the placental extract has tumor cell growth inhibition activity, we treated HepG2 cells with the placental extract and evaluated cell growth by sulforhodamine B assay. As shown in Fig. 1A, the placental extract inhibited the proliferation of HepG2 cells dose-dependently. Next, to identify the main antiproliferative components of the placental extract, we analyzed the composition of the placental extract using chromatographic methods (Fig. 1B). We performed thin-layer chromatography and HPLC (*O*-phthaldialdehyde-derivatization) analysis, and our data suggested that the final fraction contained six of amino acids including alanine (Ala), aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), serine (Ser), and threonine (Thr) (data not shown). To determine which amino acids are responsible for the inhibition of HepG2 cell growth, we performed a deletion assay. As shown in Fig. 1C, mixtures lacking Ala, Gly, Ser, or Thr alone showed almost equivalent cell growth inhibition activity relative to the Master solution consisting of the six amino acids mentioned above. In contrast, mixtures lacking Asp and Glu showed lower cell growth inhibition activity. Hence, Asp and Glu are the responsible components for the inhibition of HepG2 cell growth. Moreover, as we did not observe cell growth inhibition activity in mixtures lacking Asp or Glu alone (Fig. 1C), we evaluated various combinations of amount and treatment duration to find the most effective one. We found that the combination of 3 mM Asp with 3 mM Glu showed the highest inhibition activity (Fig. 1D).

Effect of the combination of Asp and Glu on various liver tumor cell lines

Furthermore, treatment with the combination of 6 mM Asp with 6 mM Glu for 24 h greatly reduced viability of HepG2, Huh-7, and HLE cells (38, 47, and 39%, respectively) (Fig. 2). Treatment for 48 and 72 h could further reduce cell viability below 20% (Fig. 2). Especially, the viability of HLE cells treated for 72 h was below 10%. These results indicated that the combination of Asp and Glu could inhibit growth of liver tumor cell lines at a dose- and time-dependent manner.

The combination of Asp and Glu inhibits Akt signaling pathway in HCC cell line

We hypothesized that the combination of Asp and Glu is required to regulate activities of specific protein kinases in signaling pathways essential for malignant cell growth, such as the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways (12, 13, 16–17, 25). Therefore, we examined the phosphorylation of ERK and Akt in Huh-7 cells treated with Asp and Glu using western blot. In these cells, the total levels of ERK and Akt and the level of ERK phosphorylation were unchanged, whereas the level of phospho-Akt was greatly decreased, relative to the control (Fig. 3). The selective MEK inhibitor Sorafenib or phosphoinositide 3-kinases inhibitor LY294002 used as a control, inhibited ERK or Akt phosphorylation at 10 µM or 50 µM, respectively, which are consisted with the induction of a feedback loop upon the inhibition of phosphor-ERK or -Akt signaling in this cell.

Effect of the combination of Asp and Glu in the rabbit VX2 liver tumor model

To determine the antitumor activity of the combination of Asp and Glu *in vivo*, we used the rabbit

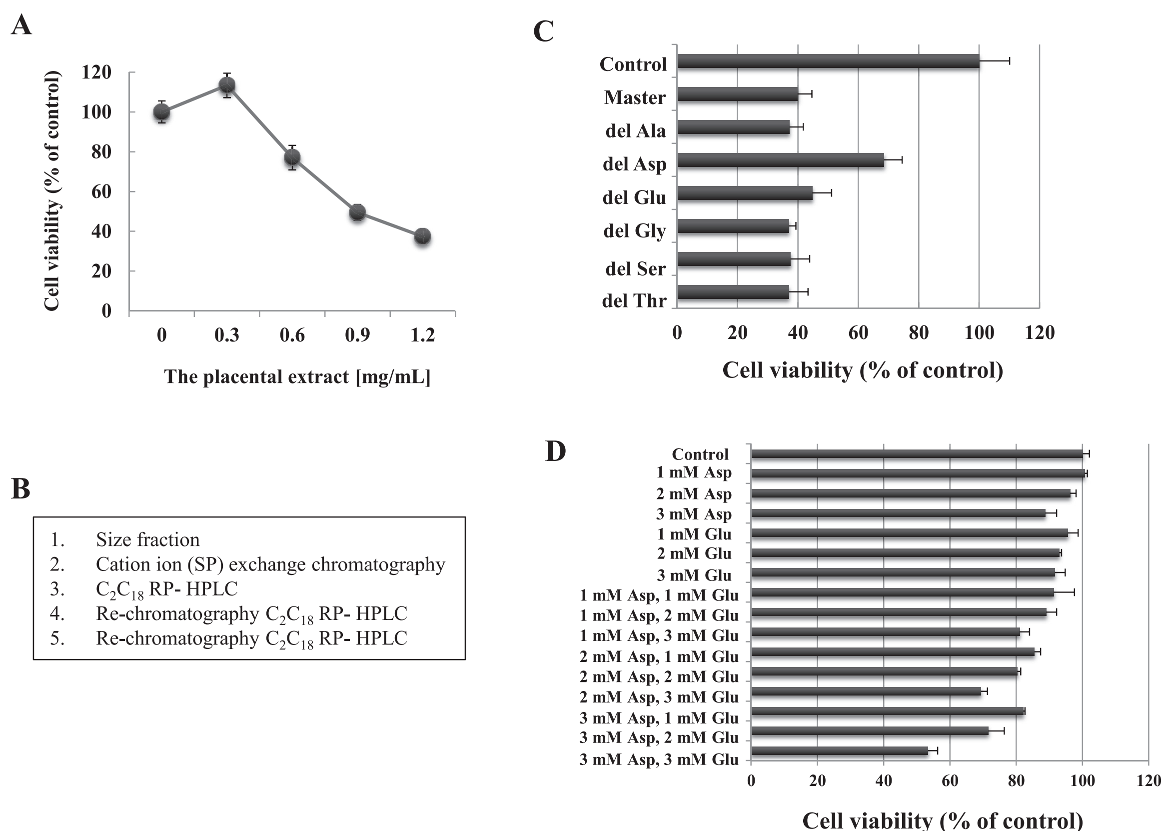

Fig. 1 Identification of anti-proliferative active components in the placental extract. Effect of the placental extract on the growth of hepatoma cells. HepG2 cells were incubated with serial dose of the placental extract or PBS (as a control) for 48 h and analyzed using the sulforhodamine B method. Data are presented as mean ± SD ($n = 3$) from 3 independent experiments (A). Chromatographic partial purification of active components in the placental extract. Summary of chromatographic methods for partial purification of active components (B). Single amino acid deletion assay to determine the components with antiproliferative activity. Amino acid mixtures with single amino acid deletion or PBS (as a control) or Master (containing Ala, Asp, Glu, Gly, Ser, or Thr alone) were added to HepG2 cells and cultured for 48 h in DMEM containing 10% FBS. Data are presented as mean ± SD ($n = 3$) from 2 independent experiments (C). Screening high potency combination of antiproliferative activity among amino acids. Combinatorial antiproliferative activity of Asp and Glu was observed. HepG2 cells were incubated with serial dose of Asp or Glu for 48 h and subjected to sulforhodamine B assay. Test samples were added to HepG2 cells and cultured for 48 h in DMEM containing 10% FBS. Data are presented as mean ± SD ($n = 3$) from 2 independent experiments (D).

VX2 liver tumor model. Lipiodol was used as a drug carrier agent to effectively induce the activity of Asp and Glu in the animals. The body weights of VX2 rabbits did not change significantly in the experimental groups (data not shown). Growth ratios of the liver tumors were determined at Day 7 after hepatic artery injection. The growth ratio of tumors in the low-dose Asp and Glu with Lipiodol group was −26.3 ± 31.7, and −33.9 ± 13.0 in the high-dose group (Table 1). There was a significant difference between the ratio of the treatment group and that of the control group. However, administration of high-dose Asp or Glu without Lipiodol showed no effect on tumor inhibition (Table 1).

DISCUSSION

In this study, we found that human placental extract suppresses the growth of human hepatocellular cells, and identified the active components to be Asp and Glu. The combination of these substances showed antiproliferative activity in a wide variety of tumor cell lines (data not shown). As deletion of Asp and Glu reduced the antiproliferative activity in the final fraction from chromatography, we concluded that these amino acids were the active components in the placental extract. However, since the deletion of these amino acids did not cause a complete loss of antiproliferative activity, we did not exclude the possibility of the presence of other active substances

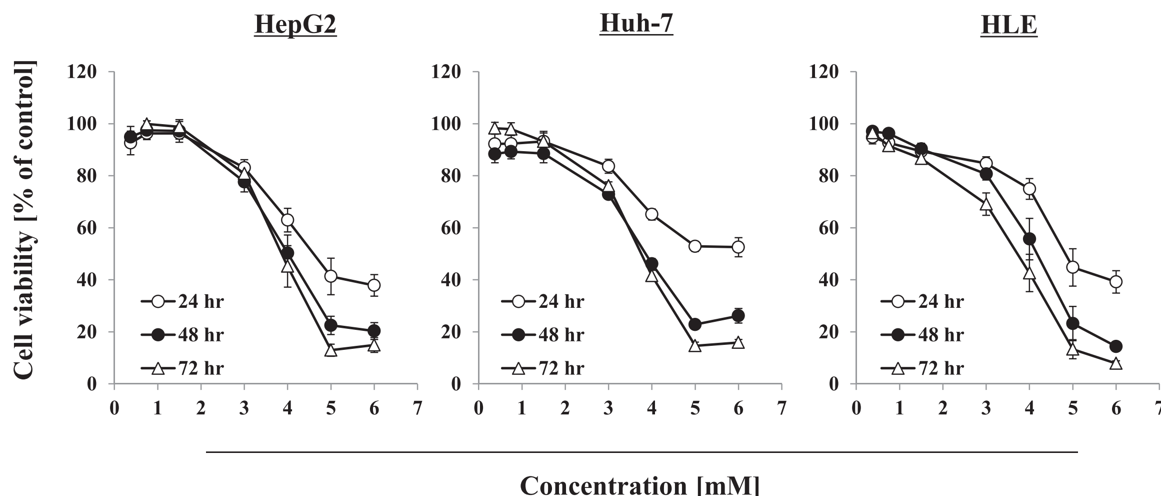

Fig. 2 Antiproliferative activity of the combination of Asp and Glu in liver tumor cell lines. The combination of Asp and Glu were added to HepG2, Huh-7, and HLE cells and cultured for 24 to 72 h in DMEM containing 10% FBS. Treatment with the combination of Asp and Glu for 24 h (open circle), 48 h (closed circle), and 72 h (open triangle). Data are presented as mean ± SD ($n = 3$) from 3 independent experiments.

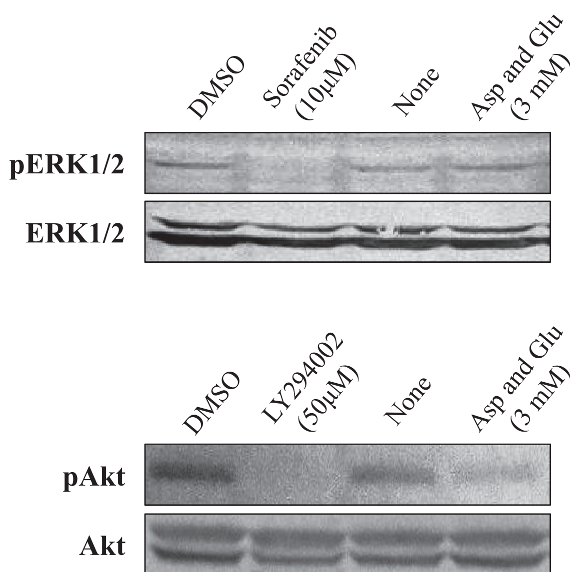

Fig. 3 The combination of Asp and Glu inhibits Akt pathway in liver tumor cells. Huh-7 cells were treated with 3 mM Asp and 3 mM Glu in DMEM containing 10% FBS for 2 h. Cells were lysed and 20 μg of protein was used for SDS-PAGE. Protein phosphorylation was detected by Western blot analysis.

in the final fraction. In fact, we observed peaks at 254 and 280 nm in the HPLC analysis of the final fraction (data not shown), which suggested the existence of molecules with an aromatic group and a nucleobase.

Amino acids function as substrates for protein synthesis, are metabolized as an energy source for protein production, and modulate numerous cellular functions (6). In addition, a number of studies have reported the anti-tumor activity of free amino acids. For example, one branched-chain amino acid (BCAA) could directly suppress HepG2 cell growth (9). Although isoleucine did not show any antiproliferative activity against colon cancer cells, it prevented tumor metastasis (24). Aspartic acid and glutamic acid are known mainly to act as neurotransmitters, and have not been reported to have tumor inhibition activity (3, 11). In our observation, although Asp or Glu alone only showed slight antiproliferative activity, their combination showed a 36% increased antiproliferative activity. These results suggested that Asp and Glu have synergistic effects on tumor suppression. Recent studies indicated that a functional *N*-methyl-D-aspartate receptor (NMDAR) was expressed in HepG2 and Huh-7 cells (20), and NMDAR-dependent signaling would be related with cancer cell growth (10, 18, 31). Therefore, Asp and Glu may function through NMDAR to inhibit tumor cell proliferation.

In our experiments, we observed that the combination of Asp and Glu inhibited Akt phosphorylation in Huh-7 cells. These results indicated that tumor cell death induced by Asp and Glu is likely through the Akt pathway. Akt belongs to the serine/threonine kinase family, and plays a crucial role in the regulatory network of the cell and affects virtually all cellular activities, especially the growth and survival of tumor cells (16–17). The PI3K/Akt/mTOR signaling pathway plays a central role in the regulation of cell

Table 1 *Effect of the combination of Asp and Glu against rabbit VX2 liver tumors*

Test samples	Mean tumor volume (cm^3)		Mean tumor growth ratio (%)
	Before administration	At experimental period	
Control (saline)	0.66 ± 0.31	3.16 ± 2.08	355.3 ± 90.1
Lipiodol only	0.68 ± 0.44	1.84 ± 0.96	263.9 ± 344.5
Low-dose Asp and Glu (0.7 mg/kg Asp and 0.8 mg/kg Glu) without Lipiodol	0.42 ± 0.11	1.92 ± 1.90	309.5 ± 313.2
High-dose Asp and Glu (1.4 mg/kg Asp and 1.5 mg/kg Glu) without Lipiodol	0.36 ± 0.16	1.94 ± 1.16	445.4 ± 167.9
Low-dose Asp and Glu (0.7 mg/kg Asp and 0.8 mg/kg Glu) with Lipiodol	0.84 ± 0.30	0.67 ± 0.50	−26.3 ± 31.7*
High-dose Asp and Glu (1.4 mg/kg Asp and 1.5 mg/kg Glu) with Lipiodol	0.53 ± 0.11	0.34 ± 0.02	−33.9 ± 13.0*

Data are presented as mean ± SE ($n = 3$). *Significant difference ($P < 0.05$) between tumor size (in two perpendicular dimensions) and treatment with test samples or control ($n = 3$ per group).

proliferation, migration, survival and angiogenesis, and is often dysregulated in HCC (12, 25), making it an attractive target for anticancer therapy. Indeed, a previous study reported that Akt phosphorylation level was up-regulated in HCC (13). In addition, perifosine, an Akt specific inhibitor, had been tested in phase II clinical trials (21). Therefore, with the inhibition activity on Akt phosphorylation, the combination of Asp and Glu may be a promising cancer therapy agent.

The combination of Asp and Glu exhibited synergistic effect on antiproliferative activity against tumor cells. Herein, the arterial chemoembolization method seems to be extremely helpful as a way of utilizing the combination of Asp with Glu *in vivo*. Iodized oil (Lipiodol) has been used as an embolic agent and a carrier of anticancer drugs. It is capable of selective accumulation and retained for a long period in hypervascular hepatic tumors. In addition, this drug delivery system enables the use of high-dose drugs (7). The combination of Asp and Glu and Lipiodol were mixed manually to prepare an oil-in-water emulsion. These mixtures tended to separate easily; however, addition of trace amounts of Tween-20 helped stabilize the mixture. There was no significant reduction in animal body weight and increase in the markers of liver or kidney injury relative to that in the control when the mixture of the combination of Asp and Glu and Lipiodol was injected into the hepatic artery (data not shown). In addition, a recent study reported greater inhibition of tumor angiogenesis in rabbits with VX2 cancer after arterial heated Lipiodol infusion compared to Lipiodol infusion (5). Since the combination of Asp and Glu is quite thermostable, it is suitable for application using this method, which would increase its clinical benefits.

In conclusion, we identified and optimized the combination of Asp and Glu as a potent tumor cell growth inhibitor from the placental extract. The combination of Asp and Glu predominantly induced necrotic cell death in HCC cell lines in a dose- and time-dependent manner, and its mode of action would be inhibition of Akt phosphorylation. Furthermore, the combination of Asp with Glu efficacy was also demonstrated *in vivo* using the rabbit VX2 liver tumor model. These results suggest that the combination of Asp and Glu may be used for induction of tumor cell death and could have clinical application as a cancer therapeutic agent.

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