Marked Inhibitory Effects of Extracts from Barley-Koji Miso Encapsulated in Hybrid Liposomes on the Growth of Human Stomach Tumor Cells in Vitro

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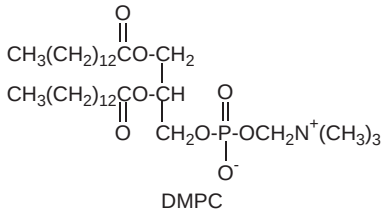
A remarkably high inhibitory effect of extracts from barley-koji miso with ethanol encapsulated in hybrid liposomes on the growth of human stomach tumor cells in vitro was obtained. Furthermore, it is suggested that ethyl linolenate should be an important antitumor agent among extracts from barley-koji miso.

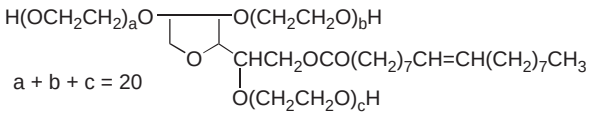
"Miso" is a traditional Japanese food produced by koji fermentation of soy bean, rice, or barley. It is well known that miso has recently attracted attention as a healthy food because of being rich in useful nutriments. In addition, Hirayama reported that the daily ingestion of miso soup significantly reduced gastric cancer risk.[1] Furthermore, it has been also reported that isoflavones included in miso have protective effects against several kinds of tumors in animal experiments.[2–4]

On the other hand, we have developed hybrid liposomes (HL)[5] and demonstrated that HL composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene alkyl ether have remarkable inhibitory effects on the growth of tumor cells in vitro and in vivo.[6] Recently, successful clinical chemotherapy with drug-free HL to patients with lymphoma has been reported.[6] Furthermore, the antitumor activities of extracts from the leaves of *Ginkgo biloba* L.[7] and the peels of *Citrus natsudaidai*[8] encapsulated in HL composed of DMPC and polyoxyethylene(20) sorbitan monolaurate were significantly enhanced as compared with those of the free extracts. These studies showed that the use of HL should be effective for improving solubilization and stabilization of hydrophobic agents in drug delivery systems.

In this study, we extracted useful components from barley-koji miso with ethanol and examined inhibitory effects of extracts encapsulated in HL composed of DMPC and polyoxyethylene(20) sorbitan monooleate (Tween 80) on the growth of human stomach tumor (GT3TKB) cells in vitro.



DMPC



Tween 80

Firstly, we extracted hydrophobic components from barley-koji miso with ethanol. Briefly, commercially available barley-koji miso (Yamauchi Honten Co., Ltd.) was digested in hot water (80 °C, 10 min). The insoluble substance in the solution was collected by suction filtration and lyophilized. The dried substance was added to ethanol at 40 °C and stirred for 4 h. After filtration of the suspension, the filtrate was evaporated and the residue was dried under reduced pressure. The extract from miso with ethanol (extract "E") was obtained as lipophilic yellow oil (yield 3.3 g/miso 100 g).

HL including extract "E" was prepared by sonication of a mixture containing DMPC, Tween 80, and extract "E" in 5% glucose solution as described previously.[8] With respect to the morphology of HL including extract "E", the hydrodynamic diameter (d_{hy}) of HL was measured by dynamic light-scattering method[8] at 37 °C. The size of HL including extract "E" was about 120 nm in diameter and stable for more than 1 month, though HL alone was gradually increased with time (Figure 1).[9] We have reported that d_{hy} of HL depends on the structure of included agents.[7] Plausibly, hydrophobic extract "E" was easily incorporated into the hydrophobic membrane domain of HL. As a result, the membrane stability was improved and the size of ca. 120 nm was kept almost constant. These results also suggest that HL including extract "E" can take an appropriate size to avoid the clearance by the reticular endothelial system (RES) in vivo.[10]

Secondly, the inhibitory effect of HL including extract "E" on the growth of GT3TKB cells in vitro was examined on the basis of WST-1 assay.[11] The tumor cells (1.0×10^{-4} viable cells/mL) were cultured for 48 h in a humidified 5% CO_2 incubator at 37 °C after adding the sample solutions. The inhibitory effect was evaluated by $A_{Mean}/A_{Control}$, where A_{Mean} and $A_{Control}$ denote the absorbance of water-soluble formazan, which was useful as an indicator of cell viability, in culture medium at 450 nm in the presence and absence of sample solutions, respectively. As shown in Figure 2, significantly high inhibitory effect of HL including extract "E" was obtained ($A_{Mean}/A_{Control} < 0.01$),

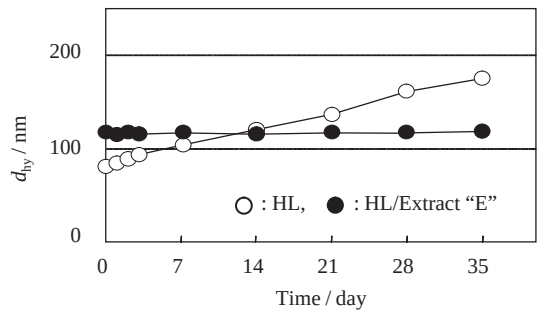


Figure 1. Time courses of d_{hy} change for HL and HL including extract "E" at 37 °C. [DMPC] = 1.0×10^{-3} M, [Tween 80] = 1.1×10^{-4} M, [Extract "E"] = 2000 μg·mL^{-1}.

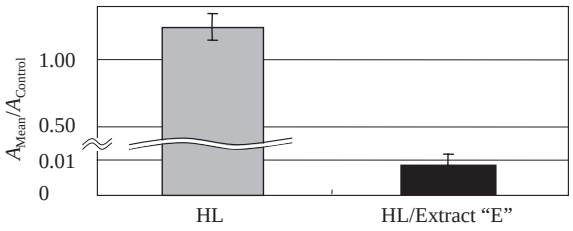


Figure 2. Inhibitory effect of HL including extract "E" on the growth of GT3TKB cells. [DMPC] = 9.1×10^{-5} M, [Tween 80] = 1.0×10^{-5} M, [Extract "E"] = 182 μg·mL^{-1}, Data presented are means; *bars*, SDs.

though no inhibitory effect of HL alone was observed. These results suggest that the extract "E" should contain effective antitumor agents.

Furthermore, GC of extract "E" was performed[12] and it was clear that the extract "E" contained several fatty acids and fatty acid ethyl esters, as shown in Figure 3. Therefore, we examined the 50% inhibitory concentrations (IC_{50}) of extract "E", fatty acids (oleic acid, linoleic acid and linolenic acid) and their ethyl esters[13] on the growth of GT3TKB cells (Table 1). Except for ethyl oleate, fairly high inhibitory effects of HL including the fatty acids or their ethyl esters were observed. Particularly, HL including ethyl linolenate was extremely attractive for the growth inhibition of GT3TKB cells. That is, the IC_{50} value (8.6 μg·mL^{-1}) of ethyl linolenate encapsulated in HL was obviously small or almost same as compared with those of antitumor isoflavones (IC_{50} of biochanin A: 7.7–17.7 μg·mL^{-1}, genistein: 6.8–18.5 μg·mL^{-1})[4] included in miso, which were tested on 7 stomach cancer lines in vitro. These observations indicate that the ethyl linolenate should be one of the important antitumor agents in barley-koji miso.

In conclusion, marked inhibitory effects on the growth of human stomach tumor cells were obtained for the first time using hybrid liposomes including the extract from barley-koji miso with ethanol. It was also attractive that ethyl linolenate should be a most important antitumor agent among extracts from barley-koji miso.

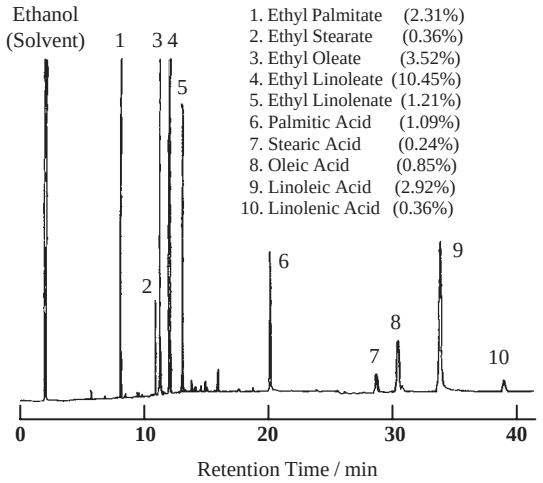


Figure 3. Gas chromatogram of extract "E". The values in parentheses are the contents of fatty acid or fatty acid ethyl ester in extract "E".

Table 1. 50% Inhibitory concentration (IC_{50}) of extract "E", fatty acids and their ethyl esters encapsulated in HL on the growth of GT3TKB cells[a]

	IC_{50}/μg·mL^{-1}
Extract "E"	65.7
Oleic acid	34.3
Linoleic acid	25.1
Linolenic acid	34.2
Ethyl oleate	>100
Ethyl linoleate	17.9
Ethyl linolenate	8.6

[a][DMPC] = 9.1×10^{-5} M, [Tween 80] = 1.0×10^{-5} M. The IC_{50} values have maximum errors of ±9.7%.

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References and Notes

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9 Differential scanning calorimetry (DSC) measurements indicated that HL alone was in the liquid crystalline state at 37 °C (The gel–liquid crystalline phase transition temperature was obtained around 22 °C). On the other hand, the phase transition temperature of HL including extract "E" was not observed in the temperature range of 0–60 °C.
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12 GC was performed on capillary column TC-FFAP using a gas chromatograph GC-353B (GL Sciences) with FID detector. The major novel peaks were assigned to the fatty acids and the ethyl esters as compared with those of standard reagents.
13 All of fatty acid ethyl esters in extract "E" were separated by PLC and inhibitory effects of those esters encapsulated in HL were examined on the basis of WST-1 assay. The $A_{Mean}/A_{Control}$ of the separated fatty acid ethyl esters was decreased to 1/5.6 of the value for extract "E".

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