Surface Modification of RGD-Liposomes for Selective Drug Delivery to Monocytes/Neutrophils in Brain

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In the present study, RGD peptide was coupled with ferulic acid (FA) liposomes for binding to monocytes and neutrophils in peripheral blood for brain targeting in response to leukocyte recruitment. Cholesterol (Ch) was esterified with succinic anhydride to introduce a carboxylic end group (Ch-COOH). Soybean phosphatidylcholine, cholesterol and Ch-COOH were in a molar ratio of 1 : 0.23 : 0.05. FA was loaded into liposomes with 80.2±5.2% entrapment efficiency (EE) using a calcium acetate gradient method since it was difficult to load FA by other methods. RGD peptide was a novel compound coupled with Ch-COOH *via* carbodiimide and *N*-hydroxysulfosuccinimide. The results of the *in vitro* flow cytometric study showed that RGD conjugation liposomes (RGD-liposomes) could bind to monocytes/neutrophils efficiently. The rats were subjected to intrastriatal microinjections of 100 μl of human recombinant IL-1β to produce brain inflammation and subsequently sacrificed after 15, 30, 60 and 120 min of administration of three formulations (FA solution, FA liposome, RGD-coated FA liposome). The body distribution results showed that RGD-liposomes could be directed to the target site, *i.e.* the brain, by cell selectivity in case of an inflammatory response. For RGD coated liposomes, the concentration of FA in brain was 6-fold higher than that of FA solution and 3-fold higher than that of uncoated liposomes. MTT assay and flow cytometry were used in the pharmacodynamic studies where it was found that FA liposomes exhibited greater antioxidant activity to FA solution on U937 cell.

Key words brain targeting; blood brain barrier; liposome; pharmacodynamic; RGD; ferulic acid

The blood–brain barrier (BBB) consists of tight intercellular junctions and plays an important role in the maintenance of the brain microenvironment.[1—3] Moreover, because of the highly lipophilic nature of the BBB, the presence of efflux transport processes, endothelial cell metabolism and plasma protein binding, many drugs fail to enter the brain following systemic administration. Hence the management of brain-related diseases with presently available therapeutic strategies is often very difficult.[4]

Evidence is accumulating that inflammation plays an important role in the pathogenesis of neurodegenerative diseases. An inflammatory reaction in the brain is found in conditions as diverse as ischemia, Alzheimer's disease and AIDS-related dementia.[5—7] In fact, in many neurological diseases, leukocytes including monocytes and neutrophils can across an intact BBB.[3,8] Thus, one of the strategies to deliver drugs to the brain under pathological conditions is to exploit these inflammatory cells as targeted delivery systems.

RGD peptide (Arg-Gly-Asp) can combine with integrin receptors which are expressed on the surface of leukocytes (neutrophils and monocytes).[9—11] The interaction between the RGD domain on the integrin molecule and the integrin receptor on leukocytes stimulates phagocytosis by polymorphonuclear cells (*e.g.* neutrophils).[12] Phagocytic and exclusive extravasation property of leukocytes makes it possible to exploit these cells as carrier system for targeted delivery.[4] It seemed possible, therefore, that RGD-liposomes could be developed for selective and preferential presentation to blood monocytes/neutrophils. Subsequently, liposomes could be taken up into the brain in response to the inflammation recruitment.[4,13] Brain targeted delivery would be possible in this elegant way.

Traditional Chinese medicines (TCMs) are treatments that are commonly advocated for a wide range of conditions in many Eastern countries and they have also become popular in the West.[14] In addition, TCMs are now being studied in great deal with reference to their low incidence of toxic side effects compared with the side effects of a number of synthetic drugs.[15] Ferulic acid (4-hydroxy-3-methoxycinnamic, FA) is one of the most important active components of several TCMs which have been used in the treatment of neurovascular and cardiovascular diseases for many years. It is known to have a wide range of pharmacological effects including antioxidant, radical scavenging, anti-apoptotic, anti-inflammatory, anticancer, antiageing, neuroprotective and antidiabetic properties,[16—20] and its antioxidant activity is particularly important.[21—23] Hence, it can be used as a promising protective agent for the treatment of neurodegenerative disorders such as Parkinson's, Alzheimer's, and Friedreich's diseases, as well as stroke[24—27] which are characterized by free radical-mediated oxidative stress, apoptosis and brain inflammation.[28—32] However, its poor penetration into brain limits its application to neurodegenerative diseases.[33] Among various approaches to improve the distribution, RGD-liposome appears to be a more promising strategy.

In the present study, an RGD-peptide was coupled to liposomes to allow site-specific drug delivery to brain. Subsequently, an evaluation was carried out of the brain targeting ability under inflammatory conditions and the pharmacological effects of FA and its preparations were also investigated.

Experimental

Chemicals and Reagents Soybean phosphatidylcholine (SPC) was a product of Taiwei Pharmaceutics Corp. (Shanghai, China). Cholesterol (Ch) was of analytical grade from Tianjin Chemical Reagent Co., Inc. (Tianjin, China). Sephadex G-50 was obtained from Pharmacia. Ferulic acid (FA) and salicylic acid were from Wanma Synthetic Drug Corp. (Zhejiang, China). RGD peptide, succinic anhydride, tBHP (*tert*-butylhydroperoxide) and Rhodamine 123 were purchased from Sigma (U.S.A.). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysulfosuccinimide (S-NHS)

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and IL-1β were from Pierce (U.S.A.). 1-Palmitoyl-2[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-Sn-glycero-3-phosphoethanolamine (NBD-PE) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.). Endostatin was a kindly gift from China Academy of Chinese Medical Sciences. OptiPrep™ was from AXIS-SHIELD PoC AS (Norway). All other chemicals were of analytical grade.

Preparation of Liposomes Briefly,[34] SPC and Ch were dissolved in chloroform and dried in a rotary evaporator under reduced pressure. The dried lipid film was redissolved in chloroform and mixed with a calcium acetate solution (120 mmol/l). Each mixture was subjected to bath-type sonication and vortexed to obtain a homogeneous w/o emulsion. The emulsion was then placed in a rotary evaporator under reduced pressure for at least 12 h to ensure the removal of organic solvent. Then, the suspension was placed in an ice bath and subjected to a 500 W ultrasonic treatment for 2 min using a high-intensity ultrasonicator (JY92-2D; Xinzhi Equ. Inst., China). Subsequently, the liposome suspension was extruded through two-stacked polycarbonate membrane of 0.22 μm at least six times.

Subsequently, each liposome suspension was eluted using a Sephadex G-50 column pre-equilibrated with Na_2SO_4 (120 mmol/l) to form a calcium acetate gradient. For the pH gradient method, the liposome suspensions were prepared in pH 7.4 PBS and eluted with pH 4.0 PBS to obtain a desired pH gradient (inside pH 7.4, outside pH 4). Then, FA was added to the suspension and incubated with the liposomes under nitrogen at 37 °C for 30 min.

FA was added to the chloroform solution including SPC and Ch for reverse-phase evaporation method (REV) (120 mmol/l $Ca(Ac)_2$, pH 7.0 acted as hydration medium for REV) and ethanol injection methods described as elsewhere.[35,36]

Determination on FA Concentration HPLC was used to determine the concentration of FA and the apparatus consisted of a mobile phase delivery pump (LC-10AD; SHIMADZU, Japan) and a UV–VIS detector (SPD-10A; SHIMADZU, Japan). Chromatography was performed on a C_{18} reverse-phase column (Thermo Quest Hypersil® ODS2 column, 150 mm×4.6 mm, 5 μ) and a Phenomenex C_{18} guard column (4 mm×3.0 mm, 5 μm, Torrance). The mobile phase was acetonitrile–0.02% glacial acetic acid (20 : 80). The ultraviolet spectrophotometry was also used to determine the concentration of FA for calculating the entrapment efficiency (EE) (spectrophotometer UV9100 Beijing, China).

The equation for calculating EE was as follows:

$$EE = W_{interior}/W_{total} \times 100\%$$

Where $W_{interior}$ represented the intraliposomal content of FA, and W_{total} represented the total content in the liposomal suspension when Triton-100 was added to the suspension. A mini-Sephadex 50 column were used to separated free drug and liposome loaded with FA for calculation of $W_{interior}$ by size exclusion chromatography.

Coupling of the RGD Peptide Cholesterol was esterified with succinic anhydride to form a carboxylic end group (Ch-COOH).[37] Liposomes were prepared as described previously. Briefly,[34] SPC, Ch and Ch-COOH (molar ratio of 1 : 0.23 : 0.05) were dissolved in chloroform and dried in a rotary evaporator under reduced pressure. The dried lipid film was redissolved in chloroform and mixed with a calcium acetate solution (120 mmol/l). The organic solvent was removed under vacuum with a rotary evaporator. The suspension was extruded through two-stacked polycarbonate membrane of 0.22 μm at least six times. Then, 10 μl 5 mM EDC and 10 μl 5 mM S-NHS were used as catalysts and added to the liposome suspension for 15 min reaction at room temperature. The excess EDC and S-NHS were removed using a Sephadex G-50 mini-column. RGD (2 mol%) was added to 2.5 ml of liposome suspension and reacted for 2 h at room temperature. The conjugation of RGD to liposomes was performed *via* coupling of the amine group on RGD and the carboxylate group on Ch-COOH.[4] Subsequently, RGD-liposome was eluted from a Sephadex G-50 mini-column pre-equilibrated with Na_2SO_4 (120 mmol/l) to form a calcium acetate gradient. FA was added to the suspension and incubated with the liposomes under nitrogen at 37 °C for 30 min. Liposomes for fluorescence assay included 1 mol% NBD-PE.

Assay of Average Size The average diameter of the plain liposomes and RGD-liposomes were measured by dynamic laser light scattering methods using a Coulter LS-230 instrument (Beckman, U.S.A.).

Freeze-Fracture Electron Microscopy Freeze-fracture electron microscopy was used as described elsewhere,[38] and a freeze-fracture device (Balzers BAF 400D) was used for fracturing.

Evaluation of Cell Binding Ability of RGD-Liposomes *in Vitro* Monocytes or neutrophils were separated from fresh human peripheral blood within 2 h of collection by the density gradient centrifugation method with OptiPrep™. Monocytes and neutrophils were incubated with NBD-labeled RGD-liposomes or NBD-labeled control liposomes for 1 h at 37 °C. For inhibition experiment, monocytes or neutrophils were pretreated with endostatin (40 μg/ml) for 30 min. Then, the unbound liposomes were removed by centrifugation, and cells were washed with PBS. Flow cytometer was used to assay the cell binding of washed, unfixed monocytes and neutrophils. A total of 10000 counts within the unlabeled cell-gated population were obtained for each sample which was tested in triplicate.

Also, NBD-labeled RGD-liposomes or NBD-labeled control liposomes were incubated with fresh human whole blood for 1 h at 37 °C and leukocyte-rich plasma (LRP) including monocytes and neutrophils was separated from the whole blood with OptiPrep™. The fluorescence intensity was determined using fluorospectrophotometer (RF-5310 PC, SHIMADZU) (λ_{ex} 458.0 nm, λ_{em} 530.0 nm).

Body Distribution Studies *In vivo* studies, the concentration of FA was determined by HPLC as described above using salicylic acid as internal standard. Male Wistar rats (12 weeks, 250±20 g) provided by the China Medical University Animals Center were divided randomly into three groups, each of three rats (the experiments complied with the requirements of the National Act on the use of experimental animals, People's Republic of China). The rats were fasted overnight but allowed free access to water before administration. Animals were subjected to intrastriatal microinjections of 100 units of human recombinant IL-1β in order to produce brain inflammation[39] and three formulations of FA solution, FA liposomes and FA RGD-liposomes (equivalent 5 mg FA/kg body weight) were given by caudal vein injection.

Determination of FA in Serum Animals were sacrificed 15, 30, 60 and 120 min after administration. Blood was collected into heparinized tubes following decapitation and separated immediately by centrifugation (10000 rpm 10 min) to collect the serum. Ten microliters internal standard (salicylic acid), 1 ml 5% trichloro acetic acid and 1 ml acetoacetate were added to 150 μl serums, followed by vortex mixing for 30 s. The mixture was then centrifuged (4000 rpm 15 min). This procedure was repeated with another 1 ml acetoacetate. The organic phase was collected and evaporated to dryness under nitrogen. Then, 200 μl mobile phase was used to redissolve the residue for determination by HPLC.

Drug Determination in Different Organs Different organs (heart, liver, spleen, lung, kidney and brain) were washed with PBS, dried, and then weighed. Every organ sample was homogenized and treated in a similar manner to serum. Whole organs were homogenized if they weighed less than 1.0 g.

Evaluation on Pharmacodynamic Actions. Cell Culture The U937 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). The cells were cultured in RPMI-1640 medium (GIBCO, NY, U.S.A.) supplemented with 10% fetal bovine serum (FBS) and 0.03% L-glutamine (GIBCO, NY, U.S.A.) and maintained at 37 °C with 5% CO_2 in a humidified atmosphere. RGD-FA liposomes for the cell assay were separated from free FA by Sephadex G-50 chromatography. The dose of liposomes in cells was calculated from the EE.

Cell Viability Assay The protective effect of ferulic acid on tBHP-challenged U937 cells was measured by MTT assay as described elsewhere.[40] The cells were dispensed in 96-well flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 2.5×10^5/ml. After 24 h incubation, they were treated with ferulic acid of various concentrations or the same concentrations of RGD-FA liposomes 1 h before 250 μM tBHP application, followed by cell culture for 12 h. Twenty microliters MTT solution (5.0×10^3 mg/l) was added to each well 4 h before the end of the incubation and the resulting crystals were dissolved in DMSO. Absorbance was measured with an ELISA reader (TECAN SPECTRA, Wetzlar, Germany) at wavelength of 490 nm. The cytotoxic effect was expressed as a relative percentage of inhibition calculated as follows:

$$\text{relative inhibition (\%)} = (A_{490\ control} - A_{490\ ferulic\ acid})/A_{490\ control} \times 100$$

Mitochondrial Transmembrane Potential ($\Delta\psi$mit) Alternation Determined by Rhodamine 123 Staining After pre-culture with ferulic acid or RGD-FA liposomes for 1 h, followed by incubation with tBHP for 12 h, cells were removed from the culture medium for staining. The culture medium was changed to PBS and washing was carried out three times. The cells were incubated with Rhodamine 123 staining stock solution (5 g/l) for 20—30 min at 37 °C. Mitochondrial transmembrane potential changes were indirectly determined by measuring the change in Rhodamine 123 fluorescence using a flow cytometer at an emission wavelength of 525 nm and an excitation wavelength of 488 nm. The samples were examined and quantified as

quickly as possible.[41)]

Statistical Analysis All results and data were confirmed by at least three independent experiments. The data were expressed as means±S.D. Statistical comparisons were made by analysis of variance (ANOVA). $p<0.05$ was considered significant.

Results and Discussion

Characterization of FA Liposomes The calcium acetate gradient method was used to load amphipathic weak acids into preformed liposomes. In this study, the optimized conditions for preparation of FA liposomes were established to achieve 80.2±5.2% EE *via* the calcium acetate gradient (lipid : drug 26 : 1 mol/mol) while it was almost impossible to load FA into liposomes by the traditional REV method, pH gradient or ethanol injection method (Fig. 1). The coating of RGD had no significantly effect on EE with the 81.0±3.7% EE of RGD-coated liposome (shown in Table 1). This may be attributed to that coating process didn't affect the stability of acetate calcium gradient and loading process of FA into liposome.

The saturation solubility of FA in intraliposomal and extraliposomal solution was determined (7.07±0.08 mg/ml in $CaAc_2$, 1.08±0.06 mg/ml in Na_2SO_4). The result showed that the intraliposomal high solubility resulted in a high EE. In most remote loading procedures, the drugs formed insoluble salt complexes in the liposomal interior.[42,43)] That suggested that the difference in solubility in the two compartments was a synergistic, rather than a decisive, effect on remote loading. In addition, for calcium acetate gradient method, the difference in calcium ion concentration across the lipid bilayers is about 0.1 M. This excess of calcium ion acts as a reservoir for more stable pH gradient which results in a significantly high EE compared with other methods. It was consistent with the previous reports.[34,42)] A representative freeze-fracture electron micrograph of FA liposomes was shown in Fig. 2. Unilamellar vesicles were present in the micrographs.

The average size of the FA liposomes and RGD liposomes were 152±0.08 nm and 155±0.06, respectively (shown in Table 1). It suggested that RGD peptide had no significant effect on their average size because RGD is a very small molecule.[4)]

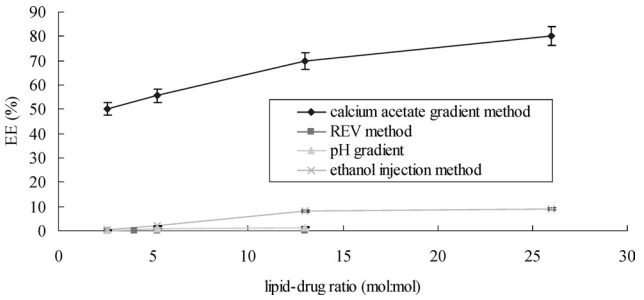

Fig. 1. The EEs of FA Liposomes *via* Various Method and Lipid-Rations at 37 °C, 30 min Incubation

Table 1. The Influence of RGD Peptide on Characteristics of FA Liposome

	Average size (nm)	Entrapment efficiency (%)
RGD-coated FA Liposome	152±0.08	80.2±5.2
Uncoated FA Liposome	155±0.06	81.0±3.7

Cell Binding Ability of RGD-Liposomes *in Vitro* In this study, blocking the amine group of RGD did not appear to be essential. There was little risk of extensive cross linking and vesicle aggregation because RGD contained only two carboxyl and two amine groups. Otherwise, if some polymerization takes place, this will produce poly RGD which will be a better ligand for monocytes/neutrophils.[4,9,10)] The addition of Sulfo-NHS stabilized the amine-reactive intermediate by converting it to an amine-reactive Sulfo-NHS ester, thus increasing the efficiency of the EDC-mediated coupling reaction. HPLC was used to determine the non-coupled peptide fraction as described elsewhere.[44)] Less than 1.5% of the added peptide was detected in free form after conjugation.

Figure 3 showed the results of cell binding ability of RGD-liposomes to monocytes/netrophils at flow cytometer. NBD-labeled RGD-coated and NBD-labeled uncoated formulations showed same level of background staining at a concentration of 270 μM total lipids (equivalent to 5.5 μM peptide accessible for binding). The results indicated a significant increase in the binding ability of RGD-coated liposomes (1.27 times and 2.06 times for monocytes and neutrophils, respectively) compared with uncoated liposome formulations.

Endostatin is an inhibitor of endothelial cell migration and has been confirmed to associate with integrins.[45—47)] In the present of endostatin, a decrease in the binding ability of RGD-liposome was observed (Fig. 3) whereas there was no

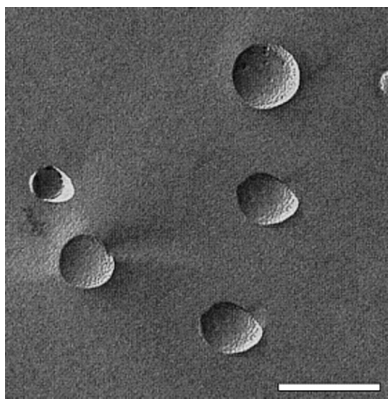

Fig. 2. Freeze-Fracture Electron Micrograph of FA Liposomes
The bar represents 200 nm.

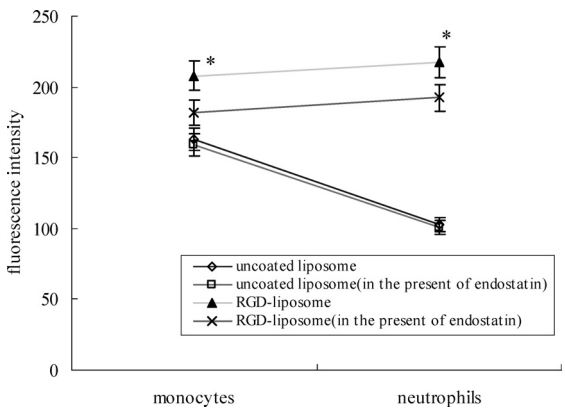

Fig. 3. Flow Cytometry Intensity Histograms of Monocytes (A) and Neutrophils (B) with Uncoated and RGD-Coated Liposomes
* Represents $p<0.05$.

significant difference for plain liposome. It demonstrated that RGD-coated liposome associated with the integrin receptors on monocytes and neutrophils selectivity.[10,11] Hence, RGD-liposome facilitated receptor-mediated endocytosis, which resulted in a higher uptake of the liposomes.[4,12]

The fluorescence intensity of LRP with NBD-labeled RGD-coated and NBD-labeled uncoated formulations was 372.17±3.49 and 100.13±3.50, respectively. It showed that 72.0% of RGD-coated liposome associated with leukocytes (monocytes and neutrophils) compared with 19.8% for plain liposome (a total fluorescence intensity of control NBD-labeled liposome 513.78±4.67). This indicated that most of RGD-coated liposomes associated with leukocytes preferentially in present of other types of cell in human whole blood. The results of the organ distribution correlated well with these *ex vivo* observations.

Body Distribution Current targeting strategies have been developed that use native proteins, antibodies or antibody fragments,[48] thereby directing liposomes toward a particular anatomical or pathological site. The amino acid sequence RGD is a major recognition system for cell adhesion and binds to integrins. Integrins are heterodimeric proteins in which each of the subunits contributes to ligand specificity and contains binding sites for the ligand. In this study, the small tripeptide was used as a model targeting ligand binding to integrin receptors on the surface of monocytes/neutrophils. These effector cells of the inflammatory response could cross an intact BBB during health and many neurological diseases and were delivered to the site of injury or infection. Leukocytes, including monocytes and neutrophils, were found in large numbers in the brain parenchyma during the "window of susceptibility" following an injection of IL-1β into the brain parenchyma.[3] Hence brain-targeting delivery was achieved.

Figure 4 showed the body distribution of FA solution, FA liposomes and RGD- coated liposomes after 15 (A), 30 (B), 60 (C), 120 (D) min of administration. As shown in Figs. 4A and B, free drug was found mainly in the kidney with a concentration of 22.7±1.83 μg/g and 25.8±1.96 μg/g after 15 and 30 min of administration, respectively. There was a clear reduction in FA concentration for FA solution in all organs after 60 min of administration according to Fig. 4C.

For the two liposome formulations, a continuous increase in FA concentration in some organs was observed after 60 min of administration (Fig. 4C) and the elimination of FA in organs showed remarkable slow compared with FA solution (Fig. 4D). There was a significant reduction in the FA kidney concentration of the two liposome formulations in Figs. 4A—D. These indicated that the body distribution of FA liposomes mainly depends on the distribution behavior of the liposomes *in vivo*. The increase of FA liposomes in liver and spleen concentration might due to absorption by the reticuloendothelial system (RES). Hence, liposomes can greatly improve the distribution behavior of FA.

As shown in Fig. 4A, very little (0.8±0.07 μg/g) of FA solution formulation reached the brain. About six times the concentration of FA (5.0±0.22 μg/g) of RGD-coated liposomes reached the brain target site *via* cell selectivity (monocytes and neutrophils). Less than twice the concentration of FA (1.5±0.05 μg/g) of uncoated liposomes reached the brain. A similar trend was obtained after 30, 60 and 120 min

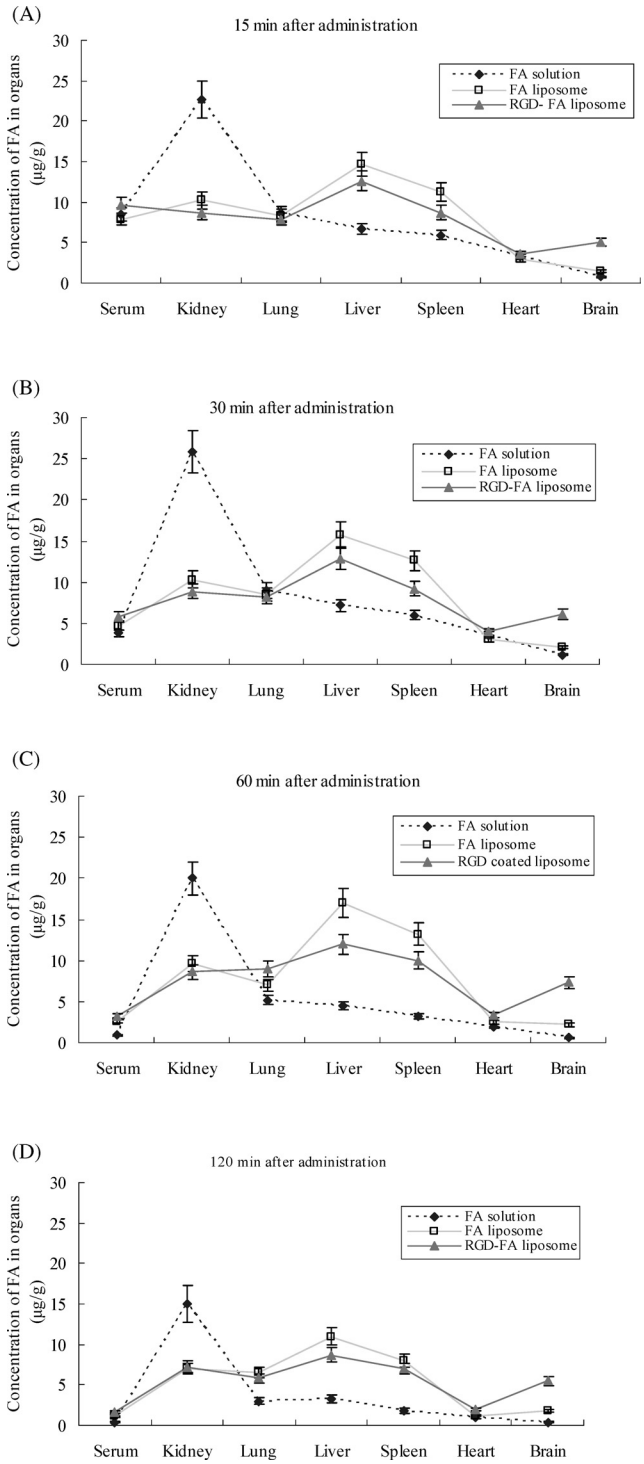

Fig. 4. The Results of Body Distribution of FA Solution, FA Liposome and RGD Coated Liposome after 15 min (A), 30 min (B), 60 min (C) and 120 min (D) of Administration

of administration according to Figs. 4B—D. The maximum brain concentration of FA (7.3±0.65 μg/g, Fig. 4C) in RGD coated liposome was 6.1-fold compared with that in FA solution (1.2±0.10 μg/g, Fig. 4B) and 3-fold in comparison to that in uncoated liposome (2.1±0.26 μg/g, Fig. 4C). Furthermore, as shown in Fig. 2D, the remarkable increase in residue of FA in brain in RGD-liposome formulation revealed that the elimination of that was significantly slow compared the other two formulations. Thus, RGD conjuga-

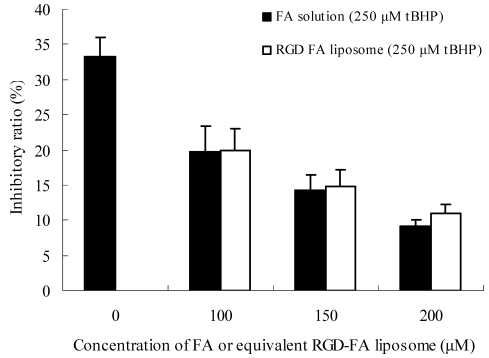

Fig. 5. The Effect of Increasing Concentrations of FA Solutions and RGD-FA Liposomes on tBHP-Induced Cytotoxicity

Results are presented at the mean±S.E.M. of at least three separate experiments.

tion FA liposome might allow a reduction in dosage.

Although FA liposomes were also accumulated on liver and spleen, the FA concentration of RGD liposomes showed a reduction in liver and spleen and a great increase in brain. It indicated that RGD-liposome could be efficiently anchored to monocytes and neutrophils to avoid the uptake of liver and spleen. Hence, RGD-liposome could significantly enhance the concentration of FA in brain. It strongly suggested that the anchor effect of RGD on monocytes and neutrophils can efficiently delivery RGD-liposome into target site.[4] In addition, uptake of liposomes by the mononuclear phagocyte system (MPS) which was detected in the central nervous system (CNS) may also contribute the delivery into brain.[3]

Pharmacodynamic Studies Oxidative stress (OS), the consequence of an imbalance of pro-oxidants and anti-oxidants in the organism, is gaining recognition as a key phenomenon in chronic illnesses like inflammatory and heart diseases, hypertension and some forms of cancer.[49] There is ample evidence of the involvement of OS in the pathogenesis of many neurological diseases. The organic hydroperoxide tBHP induces an array of cellular dysfunctions, including peroxidation of membrane lipids, depletion of GSH, perturbation of calcium ion sequestration, DNA single-strand breakage and mitochondrial damage.[50,51] In the present study, U937 cells were challenged with tBHP, which was a well-characterized model of oxidative cell injury.[52]

The MTT results were shown in Fig. 5. Twelve hours of incubation at a tBHP concentration of 250 μM reduced cell inhibitory by about 35%. The antioxidant properties of FA protected cells in a dose-dependent manner from 100 to 200 μM equivalent FA. As shown in Fig. 5, RGD-FA liposomes exhibited similar antioxidant activity to that of FA solution.

Figure 6 shows the results of flow cytometry. The organic hydroperoxide tBHP may induce mitochondrial damage related to oxidative cell injury.[48] One hundred micromolar FA and equivalent FA liposomes were used. The cell death in the tBHP, FA solution and RGD-FA liposome groups were 30.13±0.56%, 17.67±0.62%, 14.70±0.68% compared with the control group, respectively. These data indicated that the protection afforded by RGD-FA liposomes was greater than that of FA solution. The good binding ability of liposomes with cells at the target site, compared with FA solution, may result in a better protective effect.

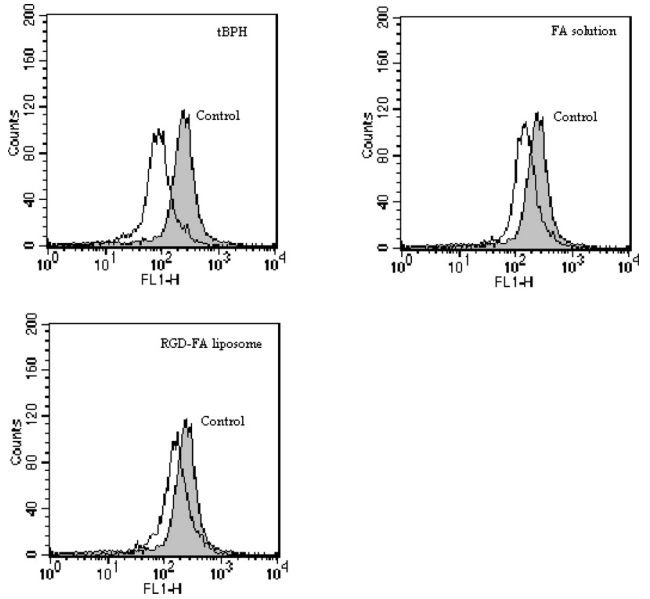

Fig. 6. Flow Cytometer Intensity Diagram of the Effect of 100 μM FA Solutions and Equivalent RGD-FA Liposomes on the Cells Challenged with 250 μM tBHP for 12 h

Conclusions

In the present study, FA liposomes were successfully prepared by the calcium acetate gradient method with 80.2±5.2% EE while it was difficult to load FA into liposomes by other methods. Intraliposomal high solubility can contribute to a high EE. RGD-liposomes showed a potent binding ability with monocytes and neutrophils in *ex vitro* study. RGD-coated liposomes exhibit brain targeting ability *in vivo* study with 6-fold concentration FA in brain compared with FA solution and 3-fold in comparison of plain liposomes. Furthermore, RGD-coated FA liposomes show a superior antioxidant activity in pharmacodynamic studies. Hence, this strategy is a promising approach because it can deliver drug directly to the inflammatory site in the brain following the recruitment of leukocytes and allow a reduction in dosage.

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