Korean J. of Chem. Eng., **13**(4), 393-398 (1996)

THE SELECTIVE DELIVERY OF ANTICANCER AGENTS USING TARGET-SENSITIVE LIPOSOMES

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(Received 29 February 1996 • accepted 10 April 1996)

Abstract – Target-sensitive (TG-S) liposomes having modified antibodies on their surface were employed to study the release of calcein and the selective delivery of the anticancer agents, doxorubicin (DOX) and methotrexate (MTX). The release of calcein from TG-S liposome occurred when the various target cells were contacted with liposomes and it was proportionally increased with the increase of antibody affinity to the target cells. Increasing the concentration of antigen molecules (major histocompatibility, MHC) on the surface of RMA-S, the release of calcein and drugs from TG-S liposomes contacting with RMA-S also rised. The destabilization of TG-S liposomes was only induced above a threshold density of surface antigen on the target cell membrane. The growth inhibition of specific target cells by the liposomal drugs was always stronger than that of the non-specific ones. For specific target cells, the IC_{50} of liposomal DOX was about 2 times greater than that of free DOX, on the while, for non-specific target cells, more than 5 times. This indicates that the liposomal drugs were transferred preferentially to the specific target cells than the non-specific ones. Based on this phenomenon, the TG-S liposomal MTX were also applied for the selective elimination of the specific target cells in the mixed culture of specific and non-specific target cells.

Key words: Liposome, Monoclonal Antibody, Targeting, Drug Delivery, Anticancer Agents

INTRODUCTION

The immunoliposomes coupled with monoclonal antibodies on their surface have been employed for the targeting of liposomes and the selective delivery of anticancer drugs [Ahmad et al., 1993; Heath et al., 1983; Leserman et al., 1980]. However, the delivery of drugs was not always successful because the release of entrapped drug did not immediately occur even after the liposomes were bound to target cells. The endocytosis of liposome may enhance the delivery of drug entrapped in liposome, but not all the cells have the endocytotic activity. To overcome this problem, target-sensitive (TG-S) liposomes had been developed by the group of Ho [1986a, 1987], which release their entrapped contents by the binding with the specific target cells, via bilayer destabilization. According to Ho et al. [1986b], at physiological conditions, stable liposomes can not be formed with the unsaturated phosphatidylethanolamine (e.g. dioleoylphosphatidylethanolamine, DOPE) since this kind of lipid rapidly undergoes phase transition to nonbilayer structure (hexagonal II phase). But a stable bilayer conformation can be obtained under physiological conditions by the addition of certain transmembrane proteins like palmitic acid acylated antibodies. As the acylated antibodies intercalated into the PE liposome can be served as not only the binding molecules to specific target cells but also the stabilizer of PE bilayer, the destabilization of PE liposomes upon binding with multivalent antigen can be induced by the local aggregation of acylated antibodies, thus these liposomes are termed as target-sensitive liposome. Ho et al. [1986a] demonstrated their TG-S liposomes could be employed for the immunoassay of virus-infected animal cells or for the efficient carrier of antiviral drug to the virus infected cells by using virus specific antibodies [Ho et al., 1987]. As the virus infected cells had relatively low endocytotic activity, the TG-S liposomes could be useful to eliminate selectively the target cells.

We have successfully prepared the TG-S liposomes and showed in the previous work [Yang et al., 1995] that the release of entrapped calcein was only observed by binding of liposomes with specific target cells. In this study, we prepared TG-S liposomes using monoclonal antibody Y3 (anti-H-$2K^{q}$, major histocompatibility, MHC) and employed these liposomes to study whether the selective delivery of drugs to specific tumor cells is possible. The specific target cells were EL-4 and RMA, which have the MHC molecule (H-$2K^{b}$) on the membrane surface. The non-specific target cells employed for comparison were L929 (H-$2K^{k}$), Yac-1 (H-$2K^{d}$), P815 (H-$2K^{d}$) and RMA-S (H-$2K^{b}$ deficient). Using the TG-S liposomes, the anticancer agents, doxorubicin or methorexate were effectively delivered to the specific target cells, while the non-specific target cells were relatively less sensitive to the drugs entrapped in TG-S liposomes comparing to the free drugs.

EXPERIMENTAL

1. Materials

Dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidic acid (DOPA), 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD chloride), calcein, sodium deoxycholate (DOC), calcium and mag-

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nesium free Hank's balanced salt solution (HBSS), N-hydroxyl succinimide ester of palmitic acid (NHSP), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and anti-mouse IgG conjugated alkaline phosphatase were purchased from Sigma. Methorexate (MTX) and doxorubicin hydrochloride (DOX) were purchased from Pharmachemie B.V. (Holand) and used as the anticancer agents.

2. Antibody Preparation

Monoclonal antibody, Y3 (anti-major histocompatibility complex class 1 of mouse, anti-H-2 K^b) was produced from hybridoma (ATCC HB176) cultured in serum free media (IMDM/F12 : 1/1 supplemented with insulin 10 μg/ml, transferrin 100 μg/ml, ethanolamine 100 μM and selenium 100 nM) and purified using hollow fiber (Microgon, USA) and DEAE-ion exchange column (1010 with LC100, Millipore, USA). The purified antibody was acylated with the NHSP at a molar ratio of 1/4 [Huang, 1985]. Using this method, according to Ho et al. [1986b], the number of palmitic acid coupled per immunoglobulin G (IgG) was 2-5 and the binding constant of IgG to target molecule was not changed. The palmitoyl IgG (p-IgG) in PBS (phosphate buffered saline, pH 8.0) containing 0.18% DOC was separated from the free palmitic acid and NHSP by Sephadex G-50 column chromatography. NBD-labelled p-IgG was also prepared by adding 20-fold molar excess of NBD chloride to p-IgG in PBS containing 0.3 % DOC [Huang, 1985].

3. Immunoliposome Preparation

Twenty μmole of DOPE and 5%(w/w) DOPA were dried and evaporated free of solvent with N_2 gas. The dry lipid was kept under vacuum for 2 hours. The appropriate p-IgG in 0.18 % DOC (0.5 ml) was added to hydrate the lipid such that the final lipid to protein ratio was 4000 : 1 (mol/mol) [Ho et al., 1986b]. For the liposome lysis experiments, 50 mM calcein was included during the hydration step as a fluorescence marker and for the drug delivery experiments, 0.5 ml of MTX (20 mg/ml) or DOX (2 mg/ml) was employed. The mixture was sonicated in a bath sonicator (Branson 2000, USA) for two 30 minutes cycles. The liposome suspension was chromatographed on a Sephadex G-50 column to remove untrapped calcein, p-IgG as well as DOC. Total entrapped calcein was measured by the addition of DOC to a final concentration of 0.12% and the percent quenching of calcein was 55-60%. The total entrapped MTX or DOX was determined by measuring the drug concentration of supernatant after the liposome solutions were centrifuged at 100,000 g for 40 min. The entrapping efficiency of MTX and DOX was about 15 and 38%, respectively.

4. Destabilization of Immunoliposomes

Calcein release experiments from TG-S liposomes were performed in a temperature controlled vessel at 37°C using 50 μl of liposome mixed with 1ml of target cell suspension. After 2 hour's contact, the fluorescence of calcein was measured at 490 and 520 nm. The percent of calcein released was calculated according to the equation;

$$\% \text{ release} = [(F - Fo)/(Ft - Fo)] \times 100$$

where Fo and F are the calcein fluorescence of the sample before and after the interaction with the cells, respectively. Ft is the total calcein fluorescence after lysis of liposome with 0.12% DOC. To determine the release rate of DOX from TG-S liposome, 300 μl (330 μg/ml) DOX solution was added into the 3 ml of cell suspension (1×10^6 cells/ml), and at the given time, 300 μl of sample was taken and centrifuged at 1,500 g. The supernatant was diluted four fold with PBS and centrifuged again at 100,000 g for 40 min. The concentration of DOX in the supernatant was determined using a fluorescence spectrophotometer at 468 and 585 nm. The amount of DOX taken by the target cells was not considered. All the experiments were duplicated.

5. Preparation of Target Cells

Specific target cells used in this work were EL4 and RMA, the T lymphomas having the surface antigen of H-$2K^b$ type, that can be bound with the Y3 monoclonal antibody. Non-specific target cells, L929 (H-$2K^k$), Yac-1 (H-$2K^l$), P815 (H-$2K^d$) and RMA-S (H-$2K^b$ deficient), were also employed for control. All the cell lines were cultured using Dulbecco's modified Eagle's medium containing 5% fetal bovine serum (FBS).

6. Determination of Antibody Binding Capacity

To determine the binding capacity of monoclonal antibody to target cells, 100 μl (20 μg/ml) of antibody labelled with fluorescence marker, NBD, was loaded on the v-shaped bottom-96 well of target cell suspensions at various concentrations. As the cell suspension contains 5% FBS, no further coating of 96 well plate with proteins such as albumin was performed. After 3 hour's contact, the cell suspension was washed three times using the calcium and magnesium free HBSS and the fluorescence of NBD bound to the target cells was measured at 460 and 554nm.

7. Induction of MHC Molecule on the Surface of RMA-S

The RMA-S cell line is defective in the ability to present endogenously synthesized antigens to class I MHC-restricted cytotoxic T lymphocytes. This defect is attributed to the inability of RMA-S to deliver antigenic peptides derived from antigens in the cytosol into the endoplasmic reticulum, where they can associate with the class I MHC [Hosken and Bevan, 1992]. However, according to Chen et al. [1994], MHC molecule can be induced by incubating the cells in a medium containing ovalbumine (OVA) peptide (257-264, Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu). We employed the same method to induce the MHC molecules on the surface of RMA-S. One hundred μl of OVA peptide solution(a gift from Korea Research Institute of Biotech.) at various concentrations was added in the v-shaped bottom-96 well of 100 μl of RMA-S cell suspension (1×10^6 cells/ml). After 4 hour's induction, the concentration of MHC molecules induced was measured by enzyme-linked immunosorbent assay (ELISA) method using Y3 antibody and anti-mouse IgG conjugated alkaline phosphatase [Johnstone and Thorpe, 1982].

8. Drug Delivery and Growth Inhibition of Target Cells

Target cells, at the initial concentration of 3000 cells/well, were pre-cultured in the 96 wells for 36 hours and the medium was replaced with one containing various formula of anticancer drugs, DOX or MTX. The cells were incubated in CO_2 incubater at 37°C for 30 minutes and the medium was replaced again with the new one. After another 36 hours of culture, the viable cell density was measured using MTT assay method [Mosmann, 1983]. The extent of drug delivery to target cells was represented by the % of cell growth inhibition as follows;

$$\text{inhibition of cell growth (\%)} = \left(1 - \frac{\text{viable cell density with drug}}{\text{viable cell density without drug}}\right) \times 100$$

All the experiments were triplicated.

RESULTS AND DISCUSSION

1. Calcein and Drug Release from TG-S Liposomes

The fluorescence of calcein entrapped in liposome is low because of the self-quenching, but that of the calcein released from the liposome is not. Using this phenomenon, the destabilization of TG-S liposomes binding with target cells could be examined. Fig. 1(A) shows the release of calcein from the TG-S liposomes containing Y3 antibody induced by the contact with the specific or non-specific target cells. Liposomes were lysed specifically in a concentration-dependent manner after exposure to the target cells. The specific target cells, EL-4 and RMA, could trigger the release of calcein, more than 80% of total entrapped amount at the cell concentration of 10^7 cells/ml within 2 hours. On the while, the non-specific target cells and RMA-S, which is deficient of the surface antigen MHC, could make the TG-S liposomes leak calcein by less than 40% with the same number of target cells. As the Y3 antibody cross-reacts with H-2 of the k, p, q, r and s haplotypes [ATCC, 1992], L929 has triggered the highest release of calcein except the specific target cells. To study the effect of the antibody affinity to target cells on the destabilization of liposomes, the binding capacity of monoclonal antibody Y3 to specific and non-specific target cells was measured using the NBD labelled antibody. The results are shown in Fig. 1(B). As we expected, the binding of Y3 to the specific target cells was higher than that to the non-specific ones. The non-specific adsorption of antibody was also negligible at the low cell concentration. Fig. 1(C) represents the relationship between the binding capacity of antibody and the amount of calcein released. The release of calcein from TG-S liposome was proportionally increased with the antibody binding capacity to target cells. This result indicates that the release of calcein is mainly due to the specific binding of antibody-coated liposomes to the target cells containing antigens [Ho et al., 1986b].

Fig. 2 shows the rate of calcein or DOX release from TG-S liposomes. With RMA at 10^6 cells/ml, 60% of calcein and 50% of DOX was released after 30-minute incubation. The contact time of the cell and liposome was fixed as 30 minutes in the following experiments based on the above results. The final amount of DOX released was not greater than 50%, which seems to be due to the two facts; as the time elapsed, the amount of DOX taken by RMA would not be negligible and the interaction between the DOX molecules and phospholipids is high enough to prevent the release of DOX into the solution. In the case of liposomes with 20% of DOPA, the release of DOX was less than 25% for 2 hours, which is the bilayer stabilizing effect of negatively charged lipids, DOPA [Pinnaduwage and Huang, 1993]. For example, the spontaneous release of DOX for liposomes with 5 and 20% DOPA in the medium of 10% serum was 8 and 1%, respectively.

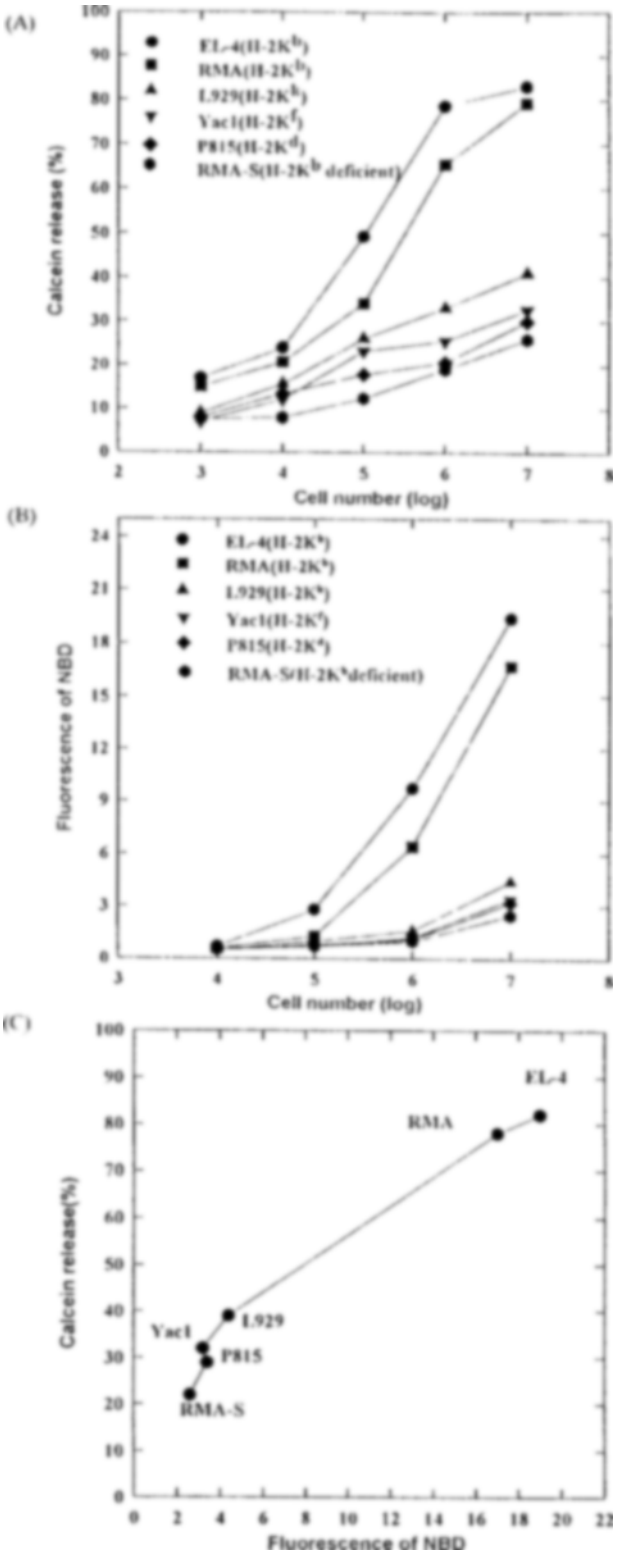

Fig. 1. The destabilization of TG-S liposomes by the interaction between the liposomes and target cells.
(A) Release of calcein from TG-S liposomes containing anti-H-2K^b antibody binding with specific (EL-4, RMA) or non-specific (L929, Yac1, P815, RMA-S) target cells (contact time: 2 hours). (B) The binding capacity of antibody to the target cells measured by NBD-labelled antibody. (C) The relationships between binding capacity of antibody and extent of calcein released from TG-S liposomes.

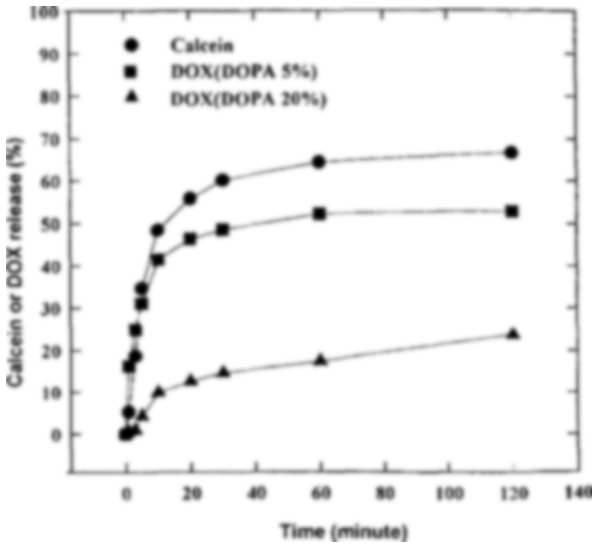

Fig. 2. The percentage of release of calcein or doxorubicin from TG-S liposomes as a function of time (RMA: 1×10^6 cells/ml).

Since the destabilization of PE liposome is induced by the local aggregation of acylated antibodies upon binding with multivalent antigen, the concentration of surface antigens of target cell is an important parameter for the release of calcein or drugs. We tested this hypothesis using RMA-S cells. Fig. 3(A) represents the result of MHC induction with OVA peptide, which was measured by ELISA at 405 nm. The number of MHC molecule was rapidly increased until 5 μg/ml of OVA peptide, then the induction rate decreased. When the optical density of ELISA of RMA was measured, the value was 0.83 (data not shown). So we could know that about 90% of MHC molecule of RMA could be induced on the surface of RMA-S with 20 μg/ml of OVA peptide. Fig. 3(B) is the result of calcein release from TG-S liposomes after the contact with RMA-S (10^6 cells/ml) previously incubated in the solution containing OVA peptide for 4 hours. Below the 3 μg/ml of OVA peptide the release of calcein was less than 20%, but 50% with the OVA peptide of 5 μg/ml. This abrupt change of releasing rate between 3 and 5 μg/ml was probably due to the requirement of threshold concentration of surface antigen molecules for the destabilization of TG-S liposomes. Pinnaduwage and Huang [1993] also showed that there was a threshold density of approximately 1mol% of epitope below which no target-induced lysis was observed. This means that multivalent binding between the liposome-bound antibodies and the surface antigens on the target cells is necessary for destabilization. Fig. 3(C) is the result of liposomal MTX (L-MTX, 100 ng/ml) delivery for 30 minutes to RMA-S after the induction of MHC. The delivery of L-MTX to target cells is monitored by the extent of cell growth inhibition. The inhibition of cell growth by L-MTX began to increase sharply after the induction with 3 μg/ml of OVA peptide, which is a little different from the result of calcein release, or the MTX release from liposome occurred earlier than that of calcein.

2. Selective Delivery of Drugs Using TG-S Liposomes

As we have confirmed that the release of calcein occurred

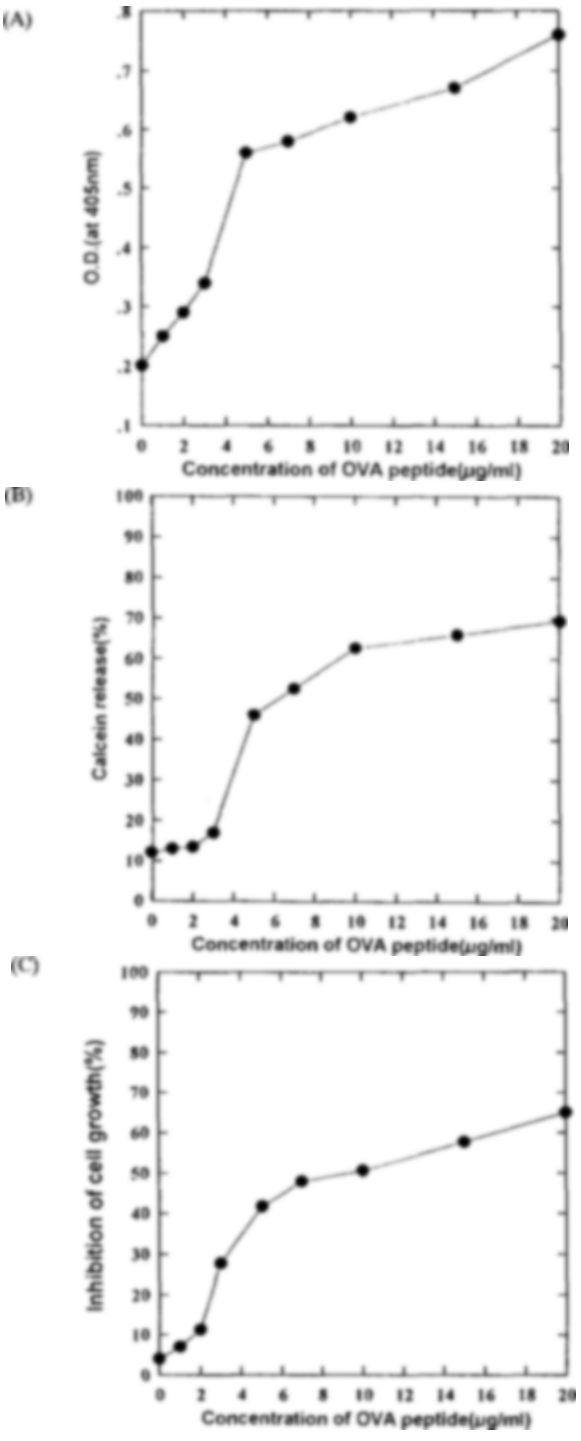

Fig. 3. The influence of surface antigen concentration of target cells on the destabilization of TG-S liposomes.
(A) Induction of MHC molecules on the membrane surface of RMA-S cells incubated in the media containing OVA peptide at various concentrations. The concentration of MHC was measured by ELISA method using anti-H-2K^b antibody (incubation time: 4 hours). (B) Release of calcein from TG-S liposomes contacted with RMA-S cells. Target cells were preincubated in OVA peptide-media (contact time: 30 minutes, cell concentration 1×10^6 cells/ml). (C) Inhibition of cell growth by the liposomal methotrexate (MTX) of RMA-S cells. Target cells were preincubated in OVA peptide-media (contact time: 30 minutes, L-MTX: 100 ng/ml).

specifically, the delivery of drug was also tested. The results are summerized in Table 1 showing the IC_{50} values of DOX or MTX for various target cells. For the specific target cells, IC_{50} of liposomal DOX was about 2 times greater than that of free DOX, whereas, for non-specific target cells, more than 5 times. With the liposomal MTX, similar experimental results were obtained. These results indicate that the liposomal durgs were transferred preferentially to the specific target cells than the non-specific ones.

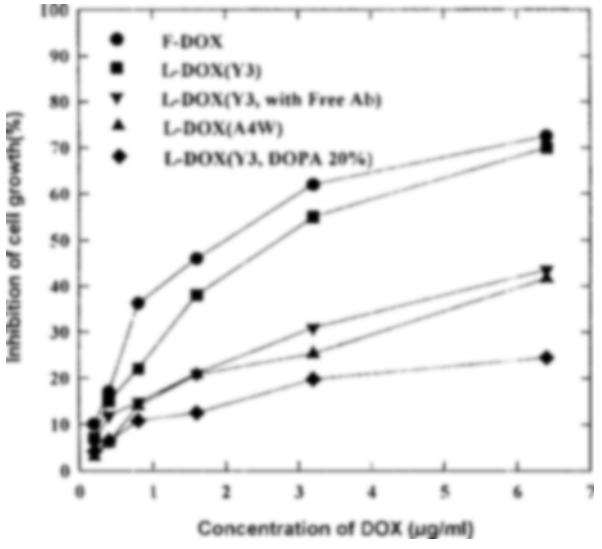

Fig. 4. Inhibition of cell growth by the free or liposomal doxorubicin (DOX) with or without free antibody, Y3 (contact time: 30 minutes).

Fig. 4 shows the result of DOX delivery to the specific target cell, EL-4. Free DOX (F-DOX) and liposomal DOX (L-DOX) with Y3 antibody were effective on the inhibition of target cell growth. On the while, if the free antibody, Y3 (20 μg/ml), was added in the medium, or if the liposome was prepared with a non-specific monoclonal antibody A4W (anti-human chorionic gonadotropin), the L-DOX did not effectively inhibit the target cell growth. This is because the soluble antibodies

Table 1. IC_{50} values of doxorubicin or methotrexate for various tumor cell lines

Target cell	IC_{50} values			
	Free-DOX (μg/ml)	Liposomal-DOX (μg/ml)	Free-MTX (ng/ml)	Liposomal-MTX (ng/ml)
EL-4	2.0	2.8	165.0	220
RMA	0.7	1.5	85.5	115.5
Yac1	1.2	>18	n.d.	n.d.
P815	1.7	>12	n.d.	n.d.
RMA-S	0.6	>12	85.5	>960
L929	16	>25	340	>960

n.d.: not determined

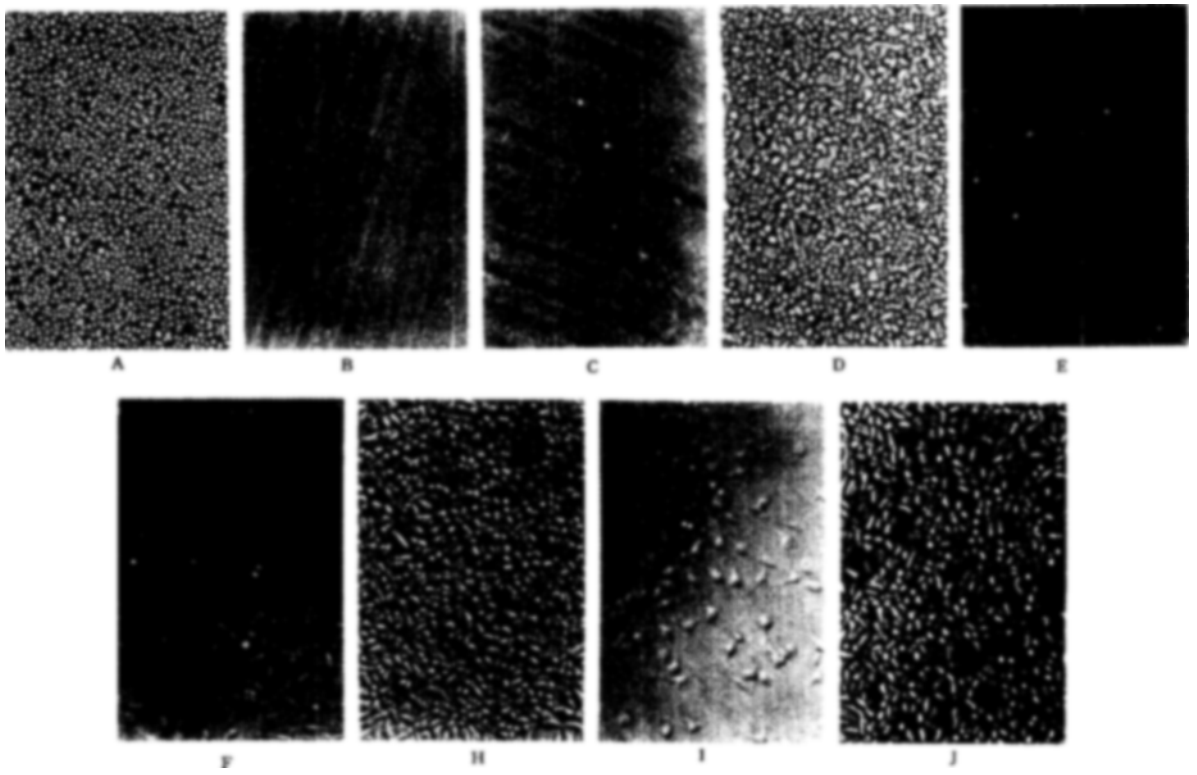

Fig. 5. Inhibition of cell growth by the free or liposomal methotrexate (MTX) in the single or mixed culture of RMA and L929. In the single culture, 3000 cells/well were cultured for 36 hours before the addition of drugs, while in the mixed culture, 1500-1500 mixed cells/well were cultured (contact time: 30 minutes).
(A, D, H) control; no addition of drug, (B, E, I) free MTX (150 ng/ml), (C, F, J) liposomal MTX (150 ng/ml), (A, B, C) RMA single culture, (D, E, F) RMA and L929 mixed culture, (H, I, J) L929 single culture

compete with the antibodies coupled to the liposome surface or because the delivery of anticancer drug from TG-S liposome occurred depending on the specificity of the antibody. When DOPA was added up to 20% in the process of liposome preparation, the drug delivery was also sharply decreased. This is due to the fact that DOPA can contribute to the stabilization of liposome structure, hence reducing the possibility of the liposome destabilization. This result also supports that the delivery of drug mainly occurred by the destabilization of liposomes, not by the endocytosis of liposomes.

3. Delivery of Drugs in the Mixed Cell Culture

In the above works, the delivery of drug was performed in the separated culture of specific or non-specific target cells. The same experiments were carried out using the mixed culture of both cells. Fig. 5 is the result of MTX delivery in the mixed culture of RMA and L929 cells. The cell growth was severely inhibited in the both type of cell culture, single or mixed culture, with the free MTX (B, E, I). On the while, with the liposomal MTX, only specific target cell was significantly inhibited (C, F, J). The cell density of L929 in mixed culture after the treatment with the liposomal MTX (F) was determined (data not shown). RMA in suspension could be easily removed because RMA is an anchorage-independent cell. According to the result, the inhibition of cell growth of non-specific target cell, L929, by the liposomal MTX was about 13%. In other words, 13% decrease of cell density was observed comparing with the single culture of L929. Together with these results, we can expect, using TG-S liposomes, the selective elimination of specific target cell in the mixed culture of specific and non-specific target cells would be possible without a severe damage on the non-specific target cells.

CONCLUSIONS

Target-sensitive (TG-S) liposomes were prepared and studied the release of calcein and the selective delivery of the anticancer agents, doxorubicin (DOX) and methotrexate (MTX) to the specific tumor cells. The release of calcein from TG-S liposome was proportionally increased with the increase of antibody affinity to the target cells but the destabilization of TG-S liposomes was only induced above a threshold density of surface antigen on the target cell membrane. It was possible *in vitro* with TG-S liposomes to deliver preferentially the anticancer angents to the specific target cells than the non-specific ones.

ACKNOWLEDGEMENT

This work was supported by the research fund of Konkuk University.

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