International Journal of Pharmaceutics, 47 (1988) 129–132
Elsevier

IJP 01590

A new confirmation of selective action of liposomes

Jordi Hernández-Borrell

Unitat de Fisicoquimica, Facultat de Farmacia, Universitat de Barcelona, Barcelona (Spain)

(Received 16 February 1988)
(Accepted 7 April 1988)

Key words: Carrier; Chemotherapy; Culture cells; Drug targeting; Liposome; Monoclonal antibody

Summary

Research in cancer chemotherapy has become a problem of selectivity. Liposomes, as a specific drug delivery system, have been largely proposed. In this paper we show proof of the selective action of liposomes, bearing Protein A on their surface, on tumoural cells in culture in the presence of monoclonal antibodies. From a pharmaceutical point of view, the detailed technology for specific liposome production was revisited, where protein adsorption on the surface was investigated by exclusion chromatography, and targeting was demonstrated by bioassay in cell culture. These were the proposed steps in order to find out the pharmacological action before carrying out further investigations in vivo using liposomes as a drug carrier in cancer therapy.

Introduction

The possibilities of using lipid vesicles or liposomes that might transport a drug to its site of action (Gregoriadis, 1981) and show immunological selectivity by the simple attachment of antibody molecules, which then recognize specific surface molecules encoded by target cells has been investigated (Huang et al., 1983).

To evaluate the extent of the immunological recognition, as well as the possible application to a selective antineoplasic action of methotrexate (MTX) on tumoral cells in culture, liposomes containing MTX and bearing Protein A on their external surface were incubated with tumoural cells (RDM4 murine thymus cells, H-2K^k-positive, HLA-negative) in the presence or absence of a specific monoclonal antibody. The MTX action was indirectly measured by the deoxy-[^{3}H]uridine test (Leserman et al., 1981).

Materials and Methods

Dipalmitoyl-phosphatidylcholine (DPPC), dipalmitoyl-phosphatidylethanolamine (DPPE), cholesterol (Chol) and methotrexate (MTX), 99% pure, were obtained from Sigma Chemicals. The purity of the lipids was checked by thin-layer chromatography. *N*-Hydroxysuccinimidyl-3-(2-pyridyldithio)propionate (SPDP), Protein A and column chromatography gels, were obtained from Pharmacia. Protein A (100 μg) was iodinated with [^{125}I]sodium iodide from New England Nuclear and chloramine-T (7 μg) and diluted with unlabeled Protein A to a final activity of 0.4 mCi/μmol. Purified carboxyfluorescein was from Kodak and dithiothreitol and organic solvents

Correspondence: J. Hernández-Borrell, Unitat de Fisicoquimica, Facultat de Farmacia, Universitat de Barcelona, 08028 Barcelona, Catalunya, Spain.

from Merck. Antibodies and the cell line was from the CIML-Marseille and from the Histology Department, Faculty of Medicine of Bilbao.

Synthesis of Protein A carrier liposomes

Three solutions were used: triethylamine (TEA) solution in chloroform (1 : 10 v/v), a SPDP solution in methanol (13.9 g/l) and DPPE in chloroform (1.5×10^{-2} M). The three solutions (0.09, 0.9 and 2.1 ml) were mixed and left at room temperature (20 °C) for 2 h. Then 2 ml of phosphate solution, pH 7.4 (PBS), was added and the emulsion washed successively with Tris-HCl buffer, pH 7.4, water and PBS, to eliminate secondary reaction products (Barbet et al., 1981). The sample was dried under nitrogen and redissolved in a mixture of benzene/chloroform (9 : 1 v/v).

The concentration of phosphatidylethanolamine 3-(2-pyridildithio)propionate (DPPE-DTP) was determined spectrophotometrically after addition of dithiothreitol.

A mixture of DPPC/CHOL/DPPE-DTP (64 : 35 : 1) at a total lipid concentration of 40 μM was then prepared and the solution evaporated under nitrogen. The residual thin layer was redissolved in 4 ml of a solution of 100 mmol/l carboxyfluorescein with 11.35 mg/ml of MTX. The suspension was then sonicated for 5 min at 80 kW under nitrogen and constant temperature (50 °C). The non-encapsulated material was separated by exclusion molecular chromatography through Sephadex G-50 (Wilschut, 1982).

The content of MTX in the liposomes was fluorometrically determined by measurement of the released carboxyfluorescein after rupture with Triton X-100 (Hernández et al., 1987). The entrapped volume was 2.8% and calculated efficiency 0.74 l/mol of phospholipid.

Liposomes were characterized by electron microscopy and light-scattering techniques, as is described elsewhere (Hernández and Pouplana, 1986, 1987) showing homogeneous distribution and an average diameter of 65 nm.

Protein A, 250 μl of 2.4×10^{-4} mmol/l solution in 10 mmol/l HEPES and 150 mmol/l NaCl, pH 7.4, was mixed with 12 μl of 10 mmol/l solution of SPDP in methanol. After 30 min of incubation at room temperature, the modified protein was chromatographically separated on a Sephadex G-25 column. The fractions with maximum radioactivity were mixed and the concentration was spectrophotometrically obtained by reduction with dithiothreitol. The yield was 90%.

Then 0.80 μl of solution of the modified Protein A (200 μg/l) was mixed with 1 ml of the liposome preparation. After 24 h at room temperature, the liposomes that had coupled the Protein A to their surface were separated from the remaining free protein by a Sepharose 4B-CL column and the yield from the coupling reaction calculated.

Cultures

RDM4 tumoural cells exhibiting the antigen K^k on their surfaces, were incubated at 37 °C in 7% CO_2/93% air in tissue culture flask with RPMI 1640 medium (Gibco) supplemented with heat-inactivated fetal calf serum (5%). Then 100 μl of the cellular suspension (25×10^3 cells) was placed in the wells of a culture plate.

Free MTX and liposomes containing MTX and carrying Protein A on their surfaces, were added to different series of wells of the culture and the specific anti-K^k antibody (H.100.5.28) (Weygang et al., 1981) added at a final concentration of 5 μg/ml. After addition of liposomes to another group of wells, a non-specific antibody (H.20.8.4) (Sharrow et al., 1984) was incorporated at a final concentration of 5 μg/ml. After 3 h of incubation, radiolabelled deoxyuridine was added to all the wells of the plates. Twelve hours later, the cells were washed and precipitated to eliminate the free radiolabelled precursor, and allow the remaining radioactivity to be counted after the residue had been dissolved in benzene.

Results and Discussion

Protein A was chosen for its anti-immunoglobulin G activity and easy determination. The fundamental strategy consisted of incubating the liposomes, bearing Protein A on their surface, with tumoural cells and the specific antibody so that the antibody could recognize the protein on the liposomes as well as the specific antigen on the

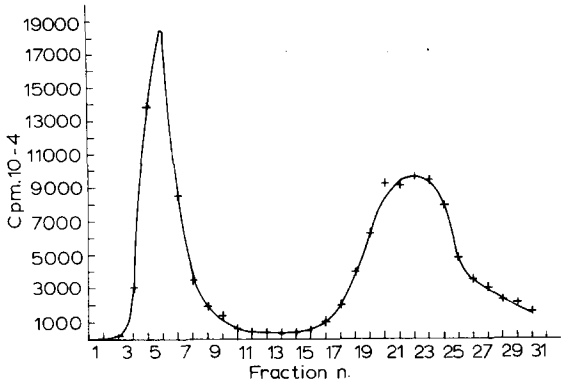

Fig. 1. Radioactivity levels of the fractions corresponding to the elution of the product between the SPDP-modified liposomes and the Protein A.

membrane of the target cell. As a result, the liposome interacts with the cell (Pagano and Weinstein, 1978), and presumably by endocytosis, the MTX can act as by mediating the reduction in radiolabeled deoxyuridine incorporation. Nevertheless, other mechanisms such as fusion between the liposome bilayer and membrane cell could be also involved.

In Fig. 1, the level of radioactivity of the fractions corresponding to the elution of the reaction product of the SPDP-modified liposomes and the Protein A is shown. The presence of 1% DPPE-DTP in the vesicle bilayer seems to be sufficient to incorporate the modified protein on the liposome surface. The presence of ^{125}I allows calculation of the coupling. The first peak corresponds to the protein carrier liposomes and the second to the free protein molecules. The calculated average yield for 10 liposome populations was 62% and the protein–lipid rate (in μmol) was $5 \times 10^{-4} \pm 15\%$.

To evaluate the cellular internalization of the encapsulated MTX, liposomes were cultured with tumoural cells. These were killed by the liposomes bearing Protein A only if the specific antibody was present in the environment. In the presence of non-specific antibodies, the cells survived. This last behaviour also shows the integrity of the liposome bilayer because no leakage of MTX could be observed.

When MTX was included, its inhibitory action on dihydrofolate reductase prevented tetrahydrofolate from being formed; therefore cellular proliferation was stopped. At the same time, the precursor deoxy-[^{3}H]uridine could not be incorporated into the cellular cytoplasm and the radioactivity levels after washing and precipitating the cells indicate a good antineoplasic action.

In Fig. 2, the percentage of ^{3}H-labelled deoxyuridine incorporation has been plotted versus the increasing concentrations of MTX. The cells were submitted to free MTX, to compare its antineoplasic action, which was significant at concentrations greater than 30 nmol with liposomes containing an equivalent concentration of MTX in their internal aqueous volume, when there was not any kind of antineoplasic action, because protein A on its own could not recognize the surface of the target cell. The inhibitory action on dihydro-

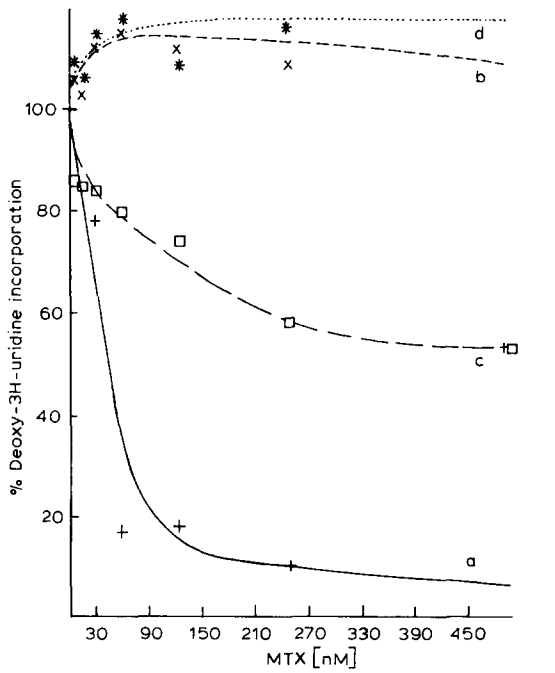

Fig. 2. Percent of labelled deoxy-[^{3}H]uridine incorporation versus the concentration of MTX (a), free MTX (b), MTX in liposomes with Protein A (c), MTX in liposomes carrying Protein A in the presence of H.100.5.28 specific antibody (d), and MTX in liposomes carrying Protein A in presence of H.20.8.4 non-specific antibody.

folate reductase could be observed when the specific antibody, H.100.5.28, was in the medium. Then the action of the MTX was demonstrated by the relative decrease in the level of deoxy-[^{3}H]uridine incorporation which is as much as 53% for a concentration of 500 nmol of MTX. With a non-specific antibody (H.20.8.4), the level of deoxy-[^{3}H]uridine was without significance (higher than 100%). From these results we found that the integrity of liposomes was in both cases guaranteed and in a first approach it seems that antineoplasic action, reflected by radioactivity levels, was a function of the liposomes bounded to the cell surface molecules encoded by RDM4.

Hence, these being the results from several experiments (14 different RDM4 cultures), we conclude that for targeting MTX-contained in liposomes, a specific antibody which recognizes H-2K^k encoded by RDM4 cells must be added to the medium. Thus, H.100.5.28 acted as a "bridge" between the Protein A-bearing liposomes and H-2K^k of the cellular surface. On the other hand, antibody can be covalently coupled to the liposome surface (Leserman et al., 1984) no protein participation being necessary. This specific stable adsorption, mediated by antibodies and surface receptors, might be the first step where liposomes can be internalized by endocytosis (Truneh et al., 1983) and where MTX acts on dihydrofolate reductase.

While the comparison between levels of incorporation shows clearly the specific behaviour when H.100.5.28 was present in the medium, differences with the levels reached with free MTX suggest a retarded action when the drug was administered in vesicles.

Briefly, we have focused on the procedure for preparation of liposomes and evaluated cytotoxicity in vitro. We expect to show in other work results on physical chemistry stability of these liposomes and pharmacokinetic behaviour.

Acknowledgements

J.H.B. gratefully acknowledges the receipt of research assistantship from C.I.R.I.T. of the Generalitat de Catalunya. Special thanks are offered to Dr. Lee D. Leserman for receiving J.H.B. in his laboratory. Also, I thank Dr. P. Machy and Ch.Noe for their support during my stay in the "Centre d'Immunologie" de Marseille-Luminy (France).

References

Barbet, J., Machy, P. and Leserman, L.D., Monoclonal antibody covalently coupled to liposomes: specific targeting to cells. *J. Supramol. Struct. Cell. Biochem.*, 16 (1981) 243–258.

Gregoriadis, G., Targeting of drugs: implication in medicine. *Lancet*, 11 (1981) 241–247.

Hernández, J. and Pouplana, R., Determinación de vesiculas lipidicas por microscopia electrónica de transmisión. *Ci. Ind. Farm.*, 5 (1986) 159–163.

Hernández, J. and Pouplana, R., Caracterización de liposomas por el método de dispersión de la luz. *II Farmaco (Ed.Pract)*, 42 (1987) 139–148.

Hernández, J., Estelrich, J. and Pouplana, R., Determination of the encapsulation efficiency in liposomes obtained by the extruder method. *J. Microencapsulation*, 4 (1987) 315–320.

Huang, A., Kennel, S.J. and Huang, L., Interactions of immunoliposomes with target cells. *J. Biol. Chem.*, 258 (1983) 14034–14140.

Leserman, L.D., Machy, P. and Barbet, J., Cell-specific drug transfer from liposomes bearing monoclonal antibodies. *Nature (Lond.)* 29 (1981) 226–228.

Leserman, L.D., Machy, P. and Barbet, J. Covalent coupling of monoclonal antibodies and Protein A to liposomes: specific interaction with cells in Vitro and in Vivo. In (Gregoriadis, Ed.), *Liposomes Technology, Vol. III*, CRC Press, Boca Raton, 1984, pp. 29–40.

Pagano, R.E. and Weinstein, J.N., Interactions of liposomes with mammalian cells. *Annu. Rev. Biophys. Bioeng.*, 7 (1978) 435–468.

Sharrow, S.O., Flaherty, L. and Sachs, K.H. Serologic cross-reactivity between class I-MHC molecules and H-2 linked differentiation antigen as detected by monoclonal antibodies. *J. Exp. Med.*, 165 (1984) 21–40.

Truneh, A., Mishal, Z., Barbet, J., Machy, P. and Leserman, L.D., Endocytosis of liposomes bound to cell surface proteins measured by flow cytofluorometry. *Biochem., J.*, 214 (1983) 189–194.

Weygang, C., Goronzy, J. and Hammerling, G.J. Recognition of polymorphic H-2 domains by T-Lymphocytes. *J. Exp. Med.*, 154 (1981) 1717–1731.

Wilschut, J. Preparation of SUV by sonication. In Leserman-Barbet (Eds.), *Liposomes Methodology*, INSERM, Paris 1982, p. 29.