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Liposome as a Carrier for Delivery of Radiomodulatory Drugs and Its Advantages in Chemo-Radiotherapy

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Asbtract

Radiomodulatory effects of free and liposome encapsulated radioprotector, MPG, and radiosensitizer, AK-2123, have been studied in mice. The drugs were intraperitoneally administered in mice 30 min prior to whole body irradiation by ^{60}Co γ-rays. Liposome encapsulated drugs, in comparison to their free forms, significantly enhanced the radiomodulatory effects in spleen and bone marrow cells. The enhancement of radiomodulation due to administration of liposomal drugs was more pronounced in bone marrow cells than in spleen cells. Liposomal MPG showed higher degree of radioprotection, whereas liposomal AK-2123 offered relatively less effect. Nonetheless, results indicate that liposomal drugs afforded enhanced radiomodulation. This may be because of delayed metabolic alteration of the encapsulated drug and enhanced concentration of drugs in bone marrow and spleen due to preferential accumulation of liposomes. As liposomes can be potentially targeted to specific tissue, this report discusses possibility of use of liposome carriers for radiosensitizing drugs for better therapeutic yields in chemo- radiotherapy of cancer.

Key words : Liposome, Liposomal Drug, MPG, AK-2123, Radiomodulation

Introduction

Radiotherapy of cancer has reached its zenith and it is obvious that radiations of different qualities and quantities as well as dose fractionation protocols can only marginally improve their present clinical efficacies. It is because radiation interaction with matter is random. Consequently, radiations cause damage to cancerous and healthy cells alike. Thus, radiations, while damaging and killing cancerous cells, also inflict damage of various kinds to normal cells which, in

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many cases, may transform these cells. This poses serious constraints on the use of radiations in therapy of cancer. In order to overcome these limitations and to improve upon clinical gains of radiotherapy of cancer, use of radiomodulatory drugs along with radiations (chemo-radiotherapy) have yielded positive therapeutic advantages. Two classes of such drugs, namely, radioprotective and radiosensitizing drugs, are relevant to chemo-radiotherapy protocols. In principle, a radioprotector shall protect normal tissues from radiation induced damages paving way for application of increased doses of radiation to kill cancerous cells more efficiently. On the other hand, a radiosensitizer shall sensitize cancerous cells so that even a lower dose of radiation could effectively kill them; thus, normal cells sustain relatively less damage. Chemo-radiotherapy, indeed, improved rate of cancer cure by radiations. However, toxicity of drugs to other tissues, lack of control on the quantity of drug in cancerous tissues after administration, non-specific sensitization of normal tissues and chemical alterations of drug in metabolism have been serious impedance to the efforts of betterment of therapeutic index of cancer chemo-radiotherapy. During the last few decades further improvements of drug efficacy in chemo-radiotherapy have relied on hybridomas and recombinant DNA technology on one hand and, on the other, on the discovery and understanding of a number of cell membrane vesicles/receptors and their interactions with ligands.

The concept of drug delivery system is based on the fact that certain carriers inherently possess or can be made to acquire selective migration to biological tissues or targets. Therefore, a drug attached onto or within this specific carrier will also be delivered to or around a specific target tissue. As expected, this approach has been shown to enhance the efficacy of drugs in a number of experimental, sub-clinical as well as in limited clinical trials[1,2].

Of the several carriers or drug delivery systems that have been studied, colloidal microspheres and liposomes have come out to be the systems with potentials to be used on humans; the latter offering several advantages over the former. We have attempted to study liposome drug delivery system as a carrier of two radiomodulatory drugs with the aim that such studies will lead to better use of these drugs in cancer chemo-radiotherapy. This report, therefore, deals only with liposome as a drug delivery system; readers are referred to a review[3] for colloidal microspheres and other drug delivery systems.

Liposomes are biomembranous sacks of 0.5 to 5 μm diameter. These are made up of lipids, phospholipids and other polar amphiphiles forming a closed concentric single (unilamellar) or multiple (multilamellar) bilayer vesicles. The vesicle can entrap with in it water and water soluble solutes as well as insoluble materials. In addition, lipid soluble or lipid bound materials can also be accommodated on the vesicle membrane. Therefore, this uniquely versatile

carrier system is very flexible vehicle for delivery of drugs of practically any chemical nature, composition, or type. The preparation can be lyophilized and stored conveniently and may be reconstituted with water. Furthermore, by using appropriately charged amphiphiles, liposomes may be tailor-made to display charge groups in its surface. This can help direct liposomes to a reciprocally charged tissue achieving limited tissue targeting. Another approach for specific tissue targeting may be tagging of antibodies on liposome surface (immuno-liposome) directing the liposomes to seek tissues with complementary antigen. Biodegradable and innocuous nature of liposomes adds to the list of advantages of liposomes as drug delivery system. The circulation time of liposomes may be varied depending on the lipid composition of the vesicle. Thus, liposomes are imminently suitable drug delivery system for use in cancer chemo-radiotherapy protocols.

Several methods of preparation of liposome vesicles are now available producing liposomes with specific characteristics such as size, surface charge, drug entrapment efficiency, fluidity, stability, drug retention time, drug release kinetics, physiological half life and pharmacokinetic behavior in different tissue sytems[2,4-6]. Despite these advantages, liposome has a serious drawback in its selective and preferential migration to tissues rich in reticuloendothelial cells like liver, spleen and bone marrow[2]. This leads to selective accumulation of normal liposomes along with its contents into these tissues. We have exploited this characteristic of liposome for simulating tissue targeting by selecting spleen and bone marrow for investigation in the present study.

In order to test suitability of liposome drug delivery system in chemo-radiotherapy, we report here an optimized and convenient method of preparation of liposomes, the entrapment efficiencies of two model radiomodulatory drugs in the liposome and the resulting radiomodulatory effects of these drugs. 2-mercaptopropionylglycine (MPG), a representative radioprotector, and N-(2'-methoxyethyl)-2-(3"-nitro-1"-triazolyl) acetamide (AK- 2123), a hypoxic cell sensitizer, have been used in this study on mice. ^{60}Co γ-radiation has been used as a source of radiation.

Materials and Methods

Chemicals

Dipalmitoyl phosphatidyl choline (DPPC), diacetylphosphate (DCP), cholesterol and trypan blue were obtained from Sigma Chemical Co., USA; dithionitrobenzoic acid from SRL, India; and Sepharose CL-4B from Pharmacia Fine Chemicals, Sweden. MPG (Tiopronine) was supplied by Santen

Pharmaceuticals Co., Japan and AK-2123 by Dr. V.T. Kagiya, Japan. All other chemicals were of highest purity grade available from Indian sources. Glass double distilled water has been used in all preparations.

Animals

Inbred, yound adult (6-8 weeks old) Swiss albino mice, colony maintained in our animal house, were used in all experiments. They were housed in polycarbonate cages with husk bedding and maintained on standard mouse pellet and drinking water *ad libitum*.

Preparation of Liposomes

Liposomes were prepared by a modified reverse-phase evaporation method reported earlier[7,8]. Briefly, 5, 2.5 and 1 mg of DPPC, cholesterol and DCP, respectively, were dissolved in 0.25 ml of chloroform. Aliquots (0.2 ml) of 1 ml of the aqueous solutions of radiomodulatory drugs to be encapsulated were added to this lipid solution while vortexing. Separation of free drug from the liposome encapsulated equal amount of drug was achieved either by centrifugation or by Sepharose CL-4B column chromatography[7]. The liposome preparations were stored refrigerated until use.

Drug Administration

Equal amount of free and liposome encapsulated drugs were administered by intraperitoneal route into mice 30 min before irradiation. The administered doses of MPG and AK-2123 were 20 mg kg^{-1} body weight and 200 mg kg^{-1} body weight, respectively.

Irradiation

Animals were acutely whole body irradiated at doses of 1, 2, 4, 6, 8, or 18 Gy in Gamma Chamber 900 (BARC, Bombay), delivering ^{60}Co γ-rays at a dose rate of less than 23 Gy min^{-1}. Animals were sacrificed for cell viability test within 60 min after irradiation.

Quantification of MPG

Assay of -SH group of MPG (Fig.1) was made to quantify the free and liposome encapsulated MPG. The method of Ellen was used with minor modification as described previously[8]. In short, 0.1 ml of test sample was added to 2.9 ml of N_2 flushed assay mixture containing 10 mM DTNB in 100 mM PBS (*p*H 7.9) and 0.1 mM EDTA. After thorough mixing, absorption was read immediately at 412 nm in a Shimadzu spectrophotometer.

Quantification of AK-2123

In the absence of a suitable chemical group on AK-2123 (Fig. 1) for spectrophotometric quantification, LASER Raman spectroscopy was employed for quantification of AK-2123. The principle of this assay lies in the presence of symmetric stretch of bonds on $-NO_2$ group of AK-2123 (Fig. 1). At a known wave number of 1313 cm_{-1} for these bonds in Raman spectroscopy, the intensity (I band) of $-NO_2$ groups of AK-2123 was measured. To avoid any errors due to sample to sample variations or instrumental errors, an internal standard of $(NH_4)_2SO_4$ was used because it also exhibits symmetric mode on $-SO_4$ with 986 cm^{-1} wave number. The relative I band of $-NO_2$ in relation to $-SO_4$ vs. concentration of AK-2123 shows a linear relationship. Using this, it has been possible to quantify AK-2123 in aqueous solution as well as in a liposome preparation.

Cell Viability Assay

The viabilities of spleen cells (SC) and bone marrow cells (BMC) of mice from different treatment groups were calculated by dye exclusion technique as

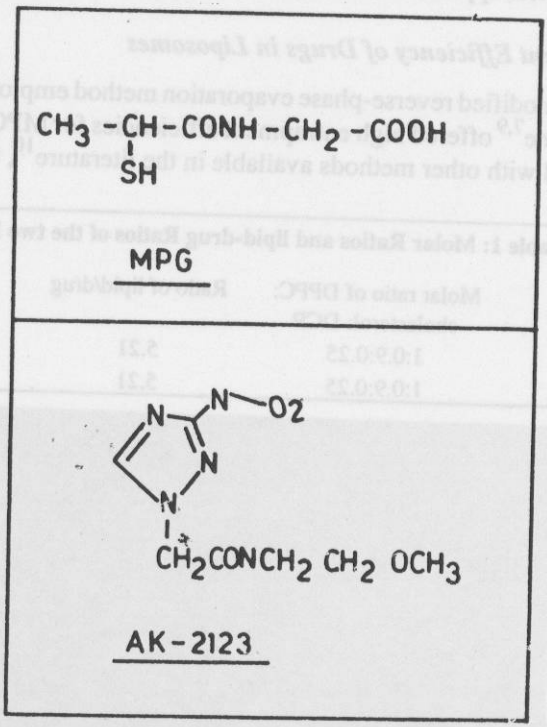

Fig.1 : Chemical structures of 2-mercaptopropionylglycine (MPG) and N-(2'-methoxyethyl)-2-(3''-nitro-1''-triazolyl) acetamide (AK- 2123).

described earlier[8]. Briefly, animals were sacrificed by cervical dislocation after irradiation and suspensions of SC and BMC in minimal essential medium were prepared. The cells were counted on a Burker chamber in phase contrast using Zena light microscope after 5 min incubation at 37 °C of cells with 1% trypan blue. The percentage of surviving cells was calculated.

Statistical Analysis

Each data point represents a minimum of 4 independent experiments each with 4 replicates. Data falling within Poissions distribution were used to calculate the mean with standard deviation (SD). Student's t-test was applied to calculate the significance of differences between different experimental groups.

Results

Preparation of Liposomes

The microphotograph of liposome preparation is shown in Fig.2. A majority of the vesicles appeared to be large-sized and the preparation was homogenous.

Entrapment Efficiency of Drugs in Liposomes

The modified reverse-phase evaporation method employed for preparation of liposome[7,9] offered high entrapment efficiencies for MPG and AK-2123. As compared with other methods available in the literature[10], this is a simple and

Table 1: Molar Ratios and lipid-drug Ratios of the two Preparations

Material	Molar ratio of DPPC: cholesterol: DCP	Ratio of lipid/drug	% entrapment
MPG	1:0.9:0.25	5.21	52
AK-2123	1:0.9:0.25	5.21	58

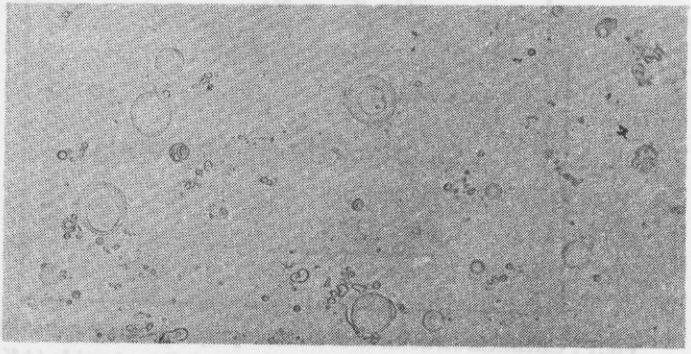

Fig.2 : Microphotograph of liposomes (Magnification X 150).

convenient method for entrapment. It shows that entrapment for MPG and AK-2123 in liposome was between 50% and 60%. The molar ratios and lipid-drug ratios for the two preparations are shown in Table 1.

Quantification of MPG

The quantification of MPG entrapped in liposomes was based on assay of -SH group of MPG (Fig. 1). The assay of -SH group was appropriate for assessment of MPG and its quantification to calculate the entrapment efficiency in liposome. The method worked for liposome entrapped MPG after lysing liposome by mild detergent treatment (2% of Triton X 100). Accordingly, it was estimated that 0.408 mg of MPG was administered to each mouse in the present investigation. Since the entrapment efficiency of MPG in liposome was about 50%, the dose of liposome encapsulated MPG was accordingly adjusted to get the same dose as free MPG.

Quantification of AK-2123

The relationship between relative I band and quantity of AK-2123 in aqueous solution was linear (Fig.3). This has been used for quantification of AK-2123 in liposomes without disintegrating liposomes. Hence, the amount of free and encapsulated AK-2123 for administration was appropriately calculated

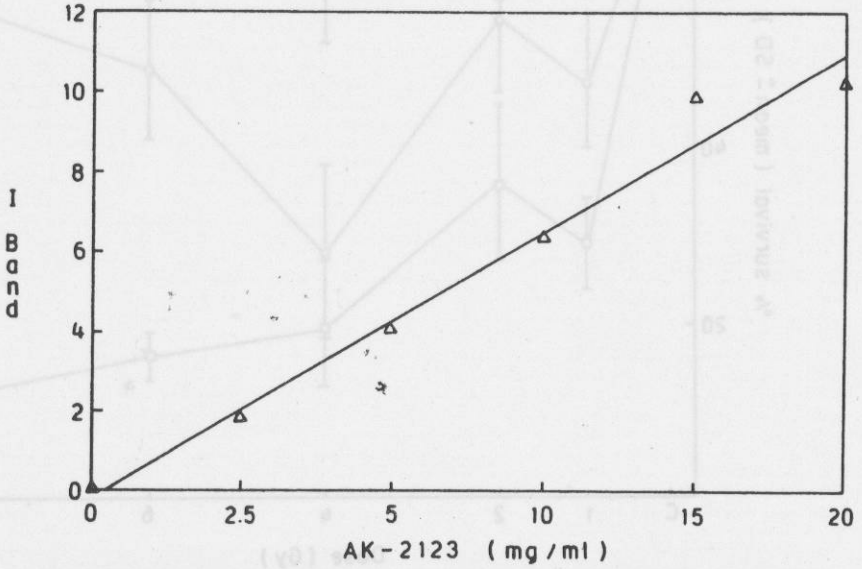

Fig.3 : The plot between quantity of AK-2123 and its relative I band showing a linear relationship (see text for details).

to deliver the equivalent effective dose of AK-2123 for radiosensitization. The method offers a very convenient way for precise quantification of AK-2123.

Effect of Radiation on MPG Treated Animals

The viabilities of SC (Fig.4) and BMC (Fig.5) decreased in a radiation dose-dependent manner. This trend was rescued by free MPG administration 30 min prior to irradiation. The presence of liposome encapsulated MPG, however, abolished the radiation dose-dependent decrease in survival of both the cell types. The enhancement of viabilities of SC and BMC was significantly higher ($p \geq 0.01$ - 0.0001) when same dose of MPG was administered as

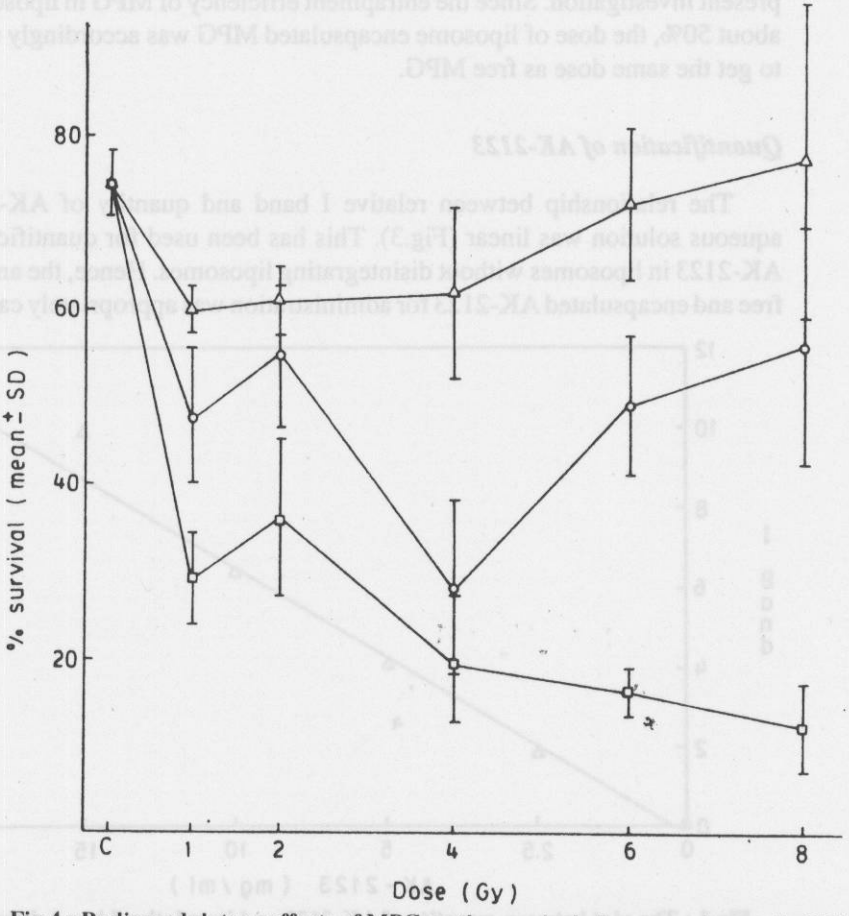

Fig.4 : Radiomodulatory effects of MPG on the survival of mouse spleen cells (SC) as revealed by dye exclusion assay (◊——◊ : radiation alone; 0——0: MPG + radiation; Δ——Δ : (liposome encapsulated MPG + radiation).

liposome encapsulated MPG prior to irradiation (Figs 4 & 5). It is to be noted that the relative protection afforded by liposome encapsulated MPG was higher at 8 Gy as compared with that at 1 Gy of gamma radiation, indicating radiation dose-dependent increase in MPG affordable radioprotection after liposome encapsulation.

Effect of Radiation on AK-2123 Treated Animals

Free AK-2123 afforded significant sensitization of cells to radiation as revealed by the radiation dose-dependent reduction in viability of SC (Fig. 6) and BMC (Fig.7). Administration of liposome encapsulated AK-2123 marginally but significantly ($p \geq 0.1\text{-}0.01$) increased sensitivity of cells to radiation. The sensitization was higher in case of BMC than SC (Figs 6 & 7).

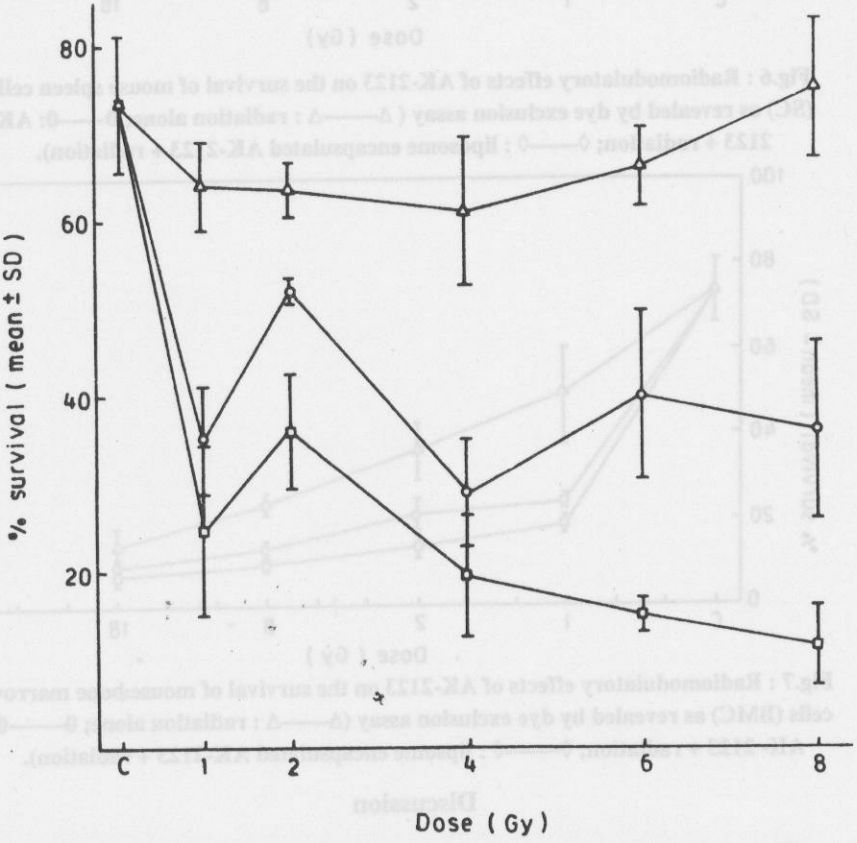

Fig.5 : Radiomodulatory effects of MPG on the survival of mouse bone marrow cells (BMC) as revealed by dye exclusion assay (δ——δ : radiation alone; 0——0: MPG + radiation ; ——— : liposome encapsulated MPG + radiation).

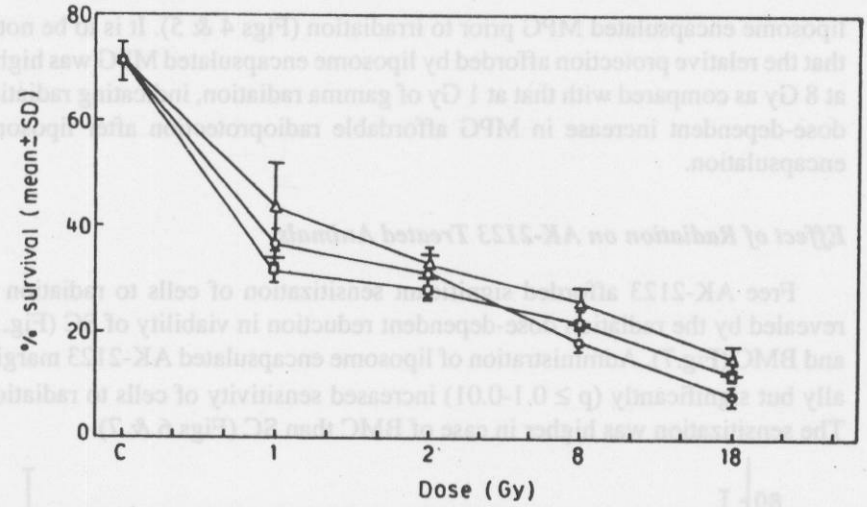

Fig.6 : Radiomodulatory effects of AK-2123 on the survival of mouse spleen cells (SC) as revealed by dye exclusion assay (Δ——Δ : radiation alone; 0——0: AK-2123 + radiation; ◊——◊ : liposome encapsulated AK-2123 + radiation).

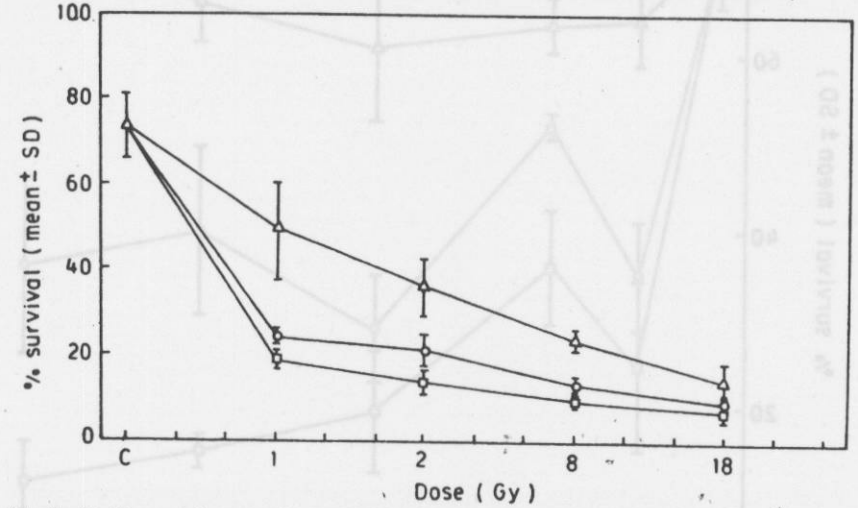

Fig.7 : Radiomodulatory effects of AK-2123 on the survival of mouse bone marrow cells (BMC) as revealed by dye exclusion assay (Δ——Δ : radiation alone; 0——0: AK- 2123 + radiation; ◊——◊ : lipsome encapsulated AK-2123 + radiation).

Discussion

The choice of two representative drugs, MPG and AK-2123, was made to test suitability of liosome encapsulation of radiomodulatory drugs and advantages it offers in chemo-radiotherapy. MPG is a known radioprotector with well

defined characteristics[11]. Similarly, AK-2123 has been shown to be of advantage over other such radiosensitizing drugs in experimental and limited clinical studies involving human patients[12,13]. These drugs were chosen to see whether liposome encapsulation, which involves contact of drug with organic solvent, alters the chemical nature of drugs. The results shown in this report (Figs 6 & 7) clearly indicate that the modified reverse-phase evaporation method of liposome preparation did not alter chemical nature of both the drugs as they remained more effective radioprotector and radiosensitizer, respectively, after liposome encapsulation. It is to be emphasized that in the method used in this report, the contact of drugs with organic solvent, chloroform, in the preparation of liposome has been small, offering this advantage; in other methods of preparation of liposome longer time of contact of organic solvents with drugs is required[10]. Furthermore, this method of liposome preparation was highly reproducible and involved mild conditions for large scale production.

The entrapment efficiency of drugs into liposome prepared by modified reverse-phase evaporation method was over 50% for the model drugs used in this investigation (Table 1), making this method highly efficient and suitable for drug encapsulation as compared with other available methods[14]. The liposome had an appropriate molar ratio of its constituents and a convenient lipid to drug ratio (Table 1).

The encapsulated radiomodifiers used in this investigation, as compared to their free forms, afforded greater radiomodulation in two tissue systems examined (Figs 4-7). The degrees of radiomodulation, however, were different for the two model drugs and for the two tissues examined. For both drugs BMC exhibited better radiomodulation (Figs 5 & 7) than SC (Figs 4 & 6). This could be due to physiological, metabolic and liposome uptake differences between BMC and SC.

The possible reasons for enhancement of radiomodulatory effects by liposomal MPG and AK-2123 could be: (1) delayed metabolic alteration of encapsulated drug, thus maintaining the active chemical forms of the drugs for a longer time, (2) reduced non- specific interaction of drugs with other metabolites which has been shown to influence radiomodulatory effects of MPG[11,15], and (3) increased concentration of drugs in these tissues due to preferential accumulation of liposomes in SC and BMC[2]. The present investigation does not shed light on the relative contributions of these three possibilities for enhancement of radiomodulation by liposome encapsulation. Nonetheless, the results show that liposome encapsulation enhanced radiomodulatory potentials of MPG and AK-2123. Gabizon[16] has suggested for DOX, an antitumor drug, that qualitative rather than quantitative differences were the cause of enhancement of effectiveness of DOX after liposome encap-

sulation. Papahadjopoulos *et al.*[17] have also reported increased efficacy of antitumor drugs after liposome encapsulation. To the best of author's knowledge, no such investigation has been carried out for radiomodulatory drugs. Therefore, This report brings out a novel finding to increase clinical efficacy of drugs for radiotherapy.

These findings may have significant impact on the clinical use of radiomodulators in cancer chemo-radiotherapy, since after liposome encapsulation the effectiveness of radiomodulatory drugs could be enhanced. This is particularly relevant for radiosensitizers which can increase clinical gains if it selectively sensitizes tumors (targets). This can be potentially achieved by using liposome drug delivery system for radiosensitizers. As emphasized earlier, liposomes could be targeted to specific cancer tissues through immunoliposomes having specific antibody against tumor antigens. Thus, radiosensitizer may be targeted to cancer tissues. This will reduce the toxicity of drugs to normal tissues, a serious problem associated with most radiosensitizers. Since cancer tissues will be sensitized, lower doses of radiation will be required to damage and kill cancer tissues. This will also reduce non- specific damages to healthy tissues. In conclusion, this approach of liposome encapsulated radiosensitizers may revolutionize the clinical practice of use of drugs in radiotherapy by enhancing efficacy of drug.

Acknowledgement

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References

1 Gregoriadis G & Florence T, Liposomes in drug delivery. *Drugs*, 45 (1993) 15-28.

2 Perez-Soler R, Liposomes as carriers of antitumor agents: towards a clinical reality. *Cancer Treat Rev*, 16 (1989) 67-82.

3 Roerdink F H and Kroon A M (editors), *Drug Carrier Systems* (Weily, Chichester), 1989.

4 Williams S S, Alosco T R, Mayhew E, Lasic D D, Martin F J and Bankert R B, Arrest of human lung xenograft growth in severe combined immuno-deficient mice using doxorubicin encapsulated in sterically stabilized liposomes. *Cancer Res*, 55 (1993) 3904-3907.

5 Rowlinson-Busza G and Epenetos A A, Targeted delivery of biologic and other antineoplastic agents. *Opin Oncol*, 4 (1992) 1142-1148.

6 Gregoriadis G, in *Limosome as Drug Carrier*, (Wiley, USA), 1988, 1-863.

7 Alam A, Bhuri S R K, Mavila A K and Singh V, Design of liposomes to improve encapsulation efficiency of gelonin and its effects on immunoreactivity and ribosome inactivating property. *Mol Cell Biochem*, 112 (1992) 107-114.

8 Sharan R N, Alam A, Chakraborty S, Saikia J R and Srivastava P N, 2-mercapto-propionylglycine affords enhanced radio protection after a liposome encapsulation. *J Radiat Res*. 36 (1995) 31-37.

9 Sharan R N, Alam A, Chakraborty S, Saikia J R and Srivastava P N, Liposome mediated delivery of 2-mercaptopropionylglycine: entrapment of MPG in liposome. *Radiosensit Newslett*, 11 (1992) 16-17.

10 Szoka F and Papahadjopoulos D, Procedure for separation of liposomes with large internal aqueous space and high capture of reverse-phase evaporation. *Proc Natl Acad Sci USA*. 75 (1978) 4194-4198.

11 Wary K K and Sharan R N, Effect of the radioprotector 2- mercaptopropionylglycine (MPG) on the radiation inactivation of catalase *in vitro*. *J Radiat Res*. 29 (1988) 104-109.

12 Kagiya V T, International co-ordinated research on the chemical modification of cancer treatment using AK-2123 - Fundamental studies, in *Radiobiological Concepts in Radiotherapy*. edited by D Bhattacharjee & B B Singh (Narosa Publishing House, New Delhi), 1995a, 135-146.

13 Kagiya V T, International co-ordinated research on the chemical modification of cancer treatment using AK-2123 — Clinical studies, in *Radioprotective Concepts in Radiotherapy*, edited by D Bhattacharjee & B B Singh (Narosa Publishing House, New Delhi), 1995b, 147-166.

14 Gregoriadis G (editor) in *Liposome Technology* (CRC Press, Boca Raton), vol. 1, 1992, 1-617.

15 Ayene S I and Srivastava P N, Radioprotective effect of 2- mercaptopropionylglycine in radiation-induced microsomal lipid peroxidation. *Int J Radiat Biol*, 48 (1985) 197-205.

16 Gabizon A A, Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long- circulating liposomes. *Cancer Res*, 52 (1992) 891-896.

17 Papahadjopoulos D, Allen T M, Gabizon A A, Mayhew E, Matthey K, Huang S K, Lee K-D, Woodle M C, Redemann C and Martin F J, Sterically stabilized liposomes: improvement in phrmacokinetics and anti tumor therapeutic efficacy. *Proc Natl Acad Sci USA*, 88 (1991) 11460-11464.