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Liposomalization of lactoferrin enhanced its anti-tumoral effects on melanoma cells

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Abstract A number of studies have reported the anti-tumoral activity of lactoferrin, a property mediated by a variety of mechanisms such as inhibitory effects on tumor cell growth, NK cell activation, and enhancement of apoptosis. Liposomes are known to be an efficient drug delivery system which can enhance the therapeutic potential of the encapsulated compounds. We have used positively charged liposomes composed of phosphatidylcholine (PC), dioleoylphosphatidylethanolamine (DOPE), cholesterol (Chol) and stearylamine (SA) (6:1:2:1 M ratio) as a carrier system for bovine iron-free Lf (ApoBLf), and compared the in vitro effect of free and liposome-entrapped ApoBLf on the growth and morphology of murine melanoma B16-F10 cells. Liposomal formulation of ApoBLf was found to enhance the capacity of the protein to inhibit the cell proliferation by affecting cell cycle progression. The effect appeared to be due to the capacity of liposomes to increase the uptake of the protein and its accumulation into cells and probably to protect it from degradation, as revealed by fluorescence microscopy and flow cytometry. Our results demonstrate the ability of liposomes to improve the anti-tumor activity of Lf and suggest that liposomal protein may have a potential therapeutic use in the prevention and/or treatment of cancer diseases.

Keywords Lactoferrin · Liposomes · Melanoma cells · Antitumoral

Abbreviations

ApoBLf	Iron free bovine lactoferrin
Chol	Cholesterol
DOPE	Dioleoylphosphatidylethanolamine
lipo-ApoBLf	Liposome-entrapped ApoBLf
PC	Phosphatidylcholine
SA	Stearylamine

Introduction

The limited efficacy of chemotherapeutic drugs, due to their non-specific toxicity, has stimulated the search for new anticancer agents.

The iron-binding glycoprotein lactoferrin (Lf), has been reported to exert in vitro and in vivo anti-tumoral activity, a property mediated through several suggested mechanisms such as inhibition of tumor cells growth, regulation of NK cells activity, inhibition of VEGF-mediated angiogenesis and enhancement of apoptosis (Ward et al. 2005; Legrand et al. 2008). The protective effect of Lf against chemically

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induced carcinogenesis, tumor growth and metastasis revealed its great potential therapeutic use in cancer disease prevention and/or treatment (Iigo et al. 2009; Rodrigues et al. 2009).

To protect the protein-based drugs from proteolysis and neutralization by serum proteins and thus enhance their therapeutic action, lipid nanostructures (liposomes) can be used as delivery system. Liposomes are able to improve the selectivity of the encapsulated drugs for tumor tissues and can accumulate in solid tumor due to the increased permeability of tumor blood vessels for circulating molecules (Torchilin 2005). Liposomalization of Lf has been demonstrated to potentiate its anti-inflammatory potential after either oral or intra-articular administration (Trif et al. 2001; Ishikado et al. 2005; Trif et al. 2007). Inhibition of tumor growth using liposomes containing naturally-occurring products such as Lf could be a possible new strategy in chemoprevention/chemotherapy.

The aim of the present study was to use positive liposomes as a carrier system for bovine iron-free Lf (ApoBLf), and to compare the in vitro effect of free and liposome-entrapped ApoBLf on the growth and morphology of murine melanoma B16-F10 cells.

Materials and methods

Reagents

BLf (kindly provided by Dr. Rex Humphrey, Tatua Dairy Cooperative, New Zealand) was rendered iron-free by dialysis against citric acid as described by Masson and Heremans (1968). The purity of protein was checked by SDS–PAGE and was higher than 90%. Contaminating endotoxin (lipopolysaccharide, LPS) was removed using Detoxi-gel (Pierce Chemical, Co.). The endotoxin content of ApoBLf preparation was less than 3 EU/ml (250 ng LPS/mg protein) as measured by the Limulus Amoebocyte Lysate (LAL) assay (Sigma Chemicals, St Louis, MO, USA).

Liposomal lipids, phosphatidylcholine (PC), dioleoylphosphatidylethanolamine (DOPE), cholesterol (Chol) and stearylamine (SA) were purchased from Sigma Chemicals. All other chemicals were of analytical grade and used as purchased.

Preparation of liposomes and Lf-loaded liposomes

Liposomes were prepared using the thin-film hydration method (Trif et al. 2001). Briefly, a mixture of PC, DOPE, Chol, SA (6:1:2:1 M ratio) was dissolved in chloroform/methanol solution (95:5) and a thin, dry film of these lipids was made by evaporating the organic solvent in a rotary evaporator (Laborota-4000, Heidolph). The film was hydrated with phosphate buffer (PBS), containing ApoBLf, by vigorous shaking. The resulted emulsion was incubated for 5 h at room temperature. Liposomes suspension containing ApoBLf (lipo-ApoBLf) was sonicated 3×30 min at 37°C. Non-entrapped protein was removed by centrifugation (1 h, 10000 rpm, 4°C) and the liposomal pellet washed twice with PBS. The amount of ApoBLf entrapped in liposomes measured by the BSA protein assay after solubilization of liposomal samples in 0.1% Triton X-100 was approximately 18%. Empty liposomes were prepared in the same way in the absence of protein. Prior to their use in cell culture experiments, empty liposomes and liposomal systems containing ApoBLf were filtered under sterile conditions through 0.45 μm filters (Millipore).

In some experiments ApoBLf was labeled with Texas Red-X succinimidyl ester (Molecular Probes) according to the manufacturer's protocol. The unbound dye was removed by gel-chromatography on Sephadex G-25. The labeled ApoBLf was included in positively charged liposomes as above.

Cells

Murine melanoma cells, B16-F10 (ECACC, Porton Down, UK) were routinely cultured in RPMI-1640 medium (EuroClone) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Biochrom). Cells were used for experiments while in the log phase of growth.

Cell proliferation and viability

Cells were seeded on 96-well plates at a density of 8×10^3 cells/well in RPMI medium and a non-radioactive proliferation assay (CellTiter96 AQueous Non-Radioactive cell proliferation assay, MTS, Promega) was performed for cell viability. Briefly,

cells were incubated with either medium or different concentrations of free ApoBLf or lipo-ApoBLf (0–1000 μg protein/ml) for 24 h. Then 20 μl of MTS were added to the wells and the plate incubated for 1 h at 37°C. The amount of formazan product, measured as the absorbance at 450 nm, is directly proportional to the number of living cells in culture. All experiments were run in triplicate. The 50% cytotoxic concentration (CC_{50}) was determined and expressed as the concentration of the compound which reduced the viability of treated cells to 50% of that of untreated cells. Cell viability was assessed in parallel by the Trypan Blue method after cell dispersion with trypsin.

Cell morphology

The morphology of cells untreated or treated with empty liposomes/ApoBLf/lipo-ApoBLf (250 μg/ml protein) was visualized by a Hemalum-Eosin technique. Briefly, cells (5×10^4) attached to glass coverslips were fixed in methanol, washed, stained with Hemalaum and then with Eosin, dehydrated in ethanol, clarified in xylene and finally mounted with Canada balsam. All samples were examined by light microscopy with a Nikon Eclipse TS100 microscope. Images (×10 and ×40) were captured with a NIS Elements software controlled digital camera.

Cell cycle analysis

Cells (2×10^4 cells/well in 24 well plates) were incubated for 24 h at 37°C with free ApoBLf and lipo-ApoBLf at a concentration of protein corresponding to the IC_{50}. After 24 h cells were washed, fixed with 100% ethanol for 2 h at 4°C, washed again and than labeled with propidium iodide (PI, 50 μg/ml). Cell cycle distribution was determined by flow cytometry using a FACS Calibur (BD, Bioscience). Data were recorded on a logarithmic scale and a minimum of 10,000 events was collected for each sample. The gating strategy excluded dead cells in FSC/SSC dot plot and the percentage of cells in G0/G1, S and G2/M cell cycle phases was determined statistically. Analysis of data was performed with CellQuest Pro Software program (BD Biosciences).

Internalization assay

Fluorescence microscopy

B16-F10 cells seeded at a density of 3×10^4 cells were incubated for 18 h at 37°C on coverslips (Menzel Glaser) in a 24 wells plate (NUNC), then treated 30 min at 37°C with 50 μg/ml free or liposome-entrapped Texas Red labeled ApoBLf, washed twice with warm PBS and finally reincubated in fresh medium for different periods of time at 37°C. Live cells were analyzed via fluorescence microscopy using a NIKON Eclipse E 600 instrument and a NIS Elements BR 3.1 software controlled NIKON Digital Sight DS-SM camera (all photos 40×). All the images shown are representative of at least two independent experiments.

FACS analysis

B16-F10 cells (1.5×10^5/ml in 12-well plates) were treated with ApoBLf, either free or liposome-entrapped at a final concentration of 1 mg/ml and incubated for different periods of time at 37°C. Cells were then detached with 0.05% trypsin-EDTA (GIBCO, Invitrogen), washed with FACS buffer (2% fetal bovine serum in PBS) and treated with BD Cytofix/Cytoperm kit (BD Biosciences) prior to incubation with 2.5 μg/ml goat anti-bovine Lf antibody (Bethyl Laboratories, Montgomery, TX, USA) for 30 min at 4°C. After washing excess primary antibody, cells were incubated with 2.5 μg/ml secondary antibody (Alexa Flour 488 donkey anti-goat IgG) (Molecular Probes) for another 30 min at 4°C. Cells were resuspended at a concentration of 10^5 cells/100 μl FACS buffer and subjected to FACS analysis, using a FACSCalibur (BD Biosciences) instrument equipped with a 488 nm argon ion laser. Fluorescence emission of samples (10000 events/sample) was recorded at 530 nm (FL1 channel) on a logarithmic scale. The gate was set on live cells and the mean fluorescence intensity (GeoMean) was determined using CellQuest Pro software (BD Biosciences). Statistical data were represented from three independent experiments.

Results and discussion

We have investigated the effect of ApoBLf, free and included into positively charged liposomes, on the

growth and morphology of murine B16-F10 cells used as a metastasis melanoma model. The iron-free form of BLf was chosen based on the reported observation that subcutaneous administration of iron-free Lf inhibited angiogenesis and suppressed tumor growth up to 21 days after B16-BL6 tumor cell inoculation (Yoo et al. 1998).

ApoBLf caused a dose-dependent cytotoxic effect on melanoma cells with 80% inhibition of cell viability at 1000 μg/ml ApoBLf (Fig. 1). Based on these data it was calculated that a protein concentration of 250 μg/ml is required to kill 50% of the melanoma cells (CC_{50}). Control experiments carried out with bovine serum albumin or bovine serum transferrin did not show any effect at concentrations up to 1000 μg/ml (data not shown). The viable counts in all cases were within 10% of those in cultures with no additions, suggesting a specific action of ApoBLf. Entrapment of ApoBLf (250 μg protein/ml) into liposomes enhanced its cytotoxic action, with cell viability dropping to 10–15%. Empty liposomes did not show any toxicity against the cell line tested (data not shown).

ApoBLf, either free or encapsulated into liposomes, also induced some morphological modifications such as changes from fusiform adherent to round, cluster-forming and floating cells (Fig. 2a–d). A reduction of cytoplasm, nuclear condensation and protrusions, as well as the presence of small apoptotic bodies, were also observed by light microscopy (Fig. 2e).

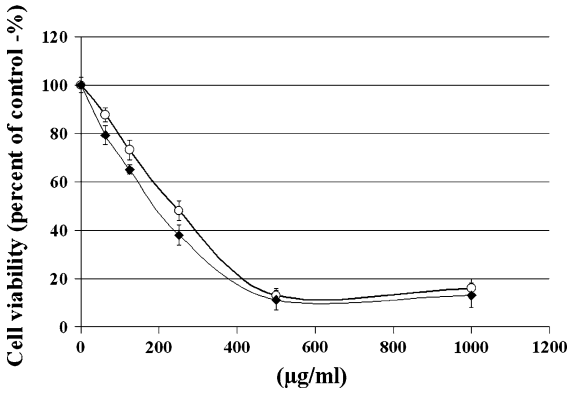


Fig. 1 Cell viability of B16-F10 cells in the presence of ApoBLf or lipo-ApoBLf. Cells were treated with different concentrations of ApoBLf (0–1000 μg/ml) either free (*circles*) or liposome-entrapped (*squares*) and after 24 h cell viability was determined using CellTiter Aqueous non-radioactive cell proliferation assay (MTS kit, Promega). *Error bars* indicate standard deviation ($n = 3$ independent experiments)

To further investigate the effect of ApoBLf and its liposomal formulation on B16-F10 cells, cell cycle analysis was performed by flow cytometry. Thus melanoma cells were incubated for 24 h with ApoBLf or lipo-ApoBLf at a protein concentration corresponding to the CC_{50}. FACS analysis of cell cycle distribution revealed a growth arrest at G0-G1 (quiescent) phase from 54% in control (untreated cells) to 65% in cells treated with ApoBLf (Fig. 3a, b). This was associated with a decrease in the S (DNA synthesis) phase of the cells from 22 to 15%, suggesting that B16-F10 DNA synthesis was affected in media containing ApoBLf. The entrapment of the protein into liposomes enhanced the effect on cell cycle phases, only 11% of cells treated with lipo-ApoBLf being present in S-phase (Fig. 3d). Empty liposomes treatment did not induced any significant modifications on cell cycle (Fig. 3c).

The cell cycle is one of the factors which play a key role in tumor progression. Each phase of the cell cycle is controlled by various cyclin proteins and Lf was reported to induce G1 arrest in breast carcinoma cells by modulating the expression and activity of key regulatory proteins, G1 cyclin-depended kinases (Damiens et al. 1999).

Our data revealed that ApoBLf inhibits B16-F10 cell proliferation, an effect which could be enhanced by its encapsulation into liposomes. In vitro studies have previously shown that cationic liposomes are cytotoxic or induce apoptosis in macrophages and immune cells (Takano et al. 2003). The effect was found to be related to the concentration of the SA and Chol components in the lipid formulation and involved the production of reactive oxygen species (Nguyen et al. 2007). However, in our experiments the lipid concentration (100 μM) used for liposomes preparation was not cytotoxic to B16-F10 cells as revealed by the results of the MTS assay.

The enhancement of Lf's cytotoxicity when entrapped into liposomes observed in our study could be due to the capacity of liposomes to increase the intracellular accumulation of protein and probably to protect it from lysosomal or proteasomal degradation. In this regard cellular uptake of ApoBLf labeled with Texas Red versus its liposomal formulation was comparatively studied in experiments using fluorescent microscopy. To follow intracellular

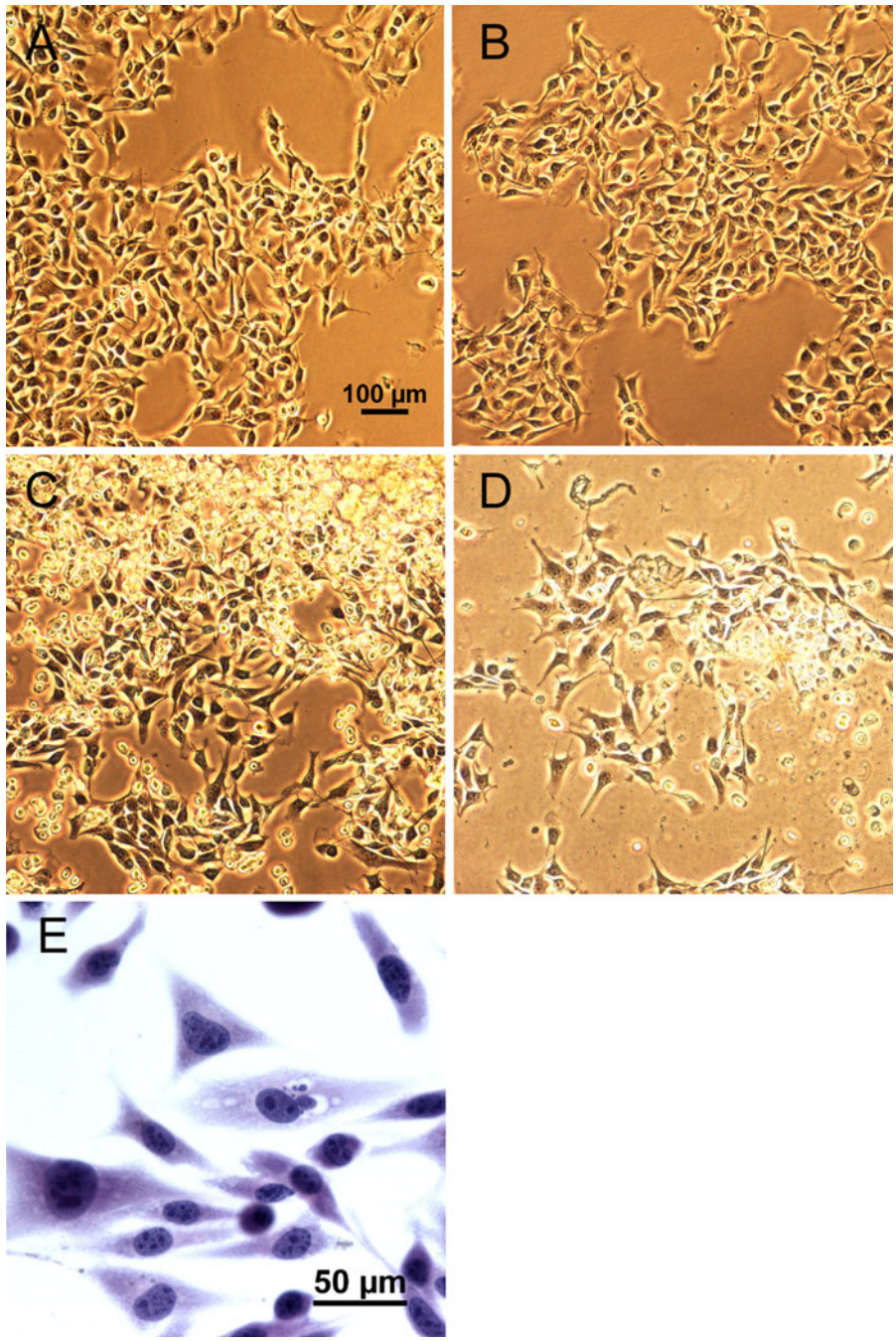


Fig. 2 Morphological changes in B16-F10 murine melanoma cells. Cells attached on coverslips were cultured for 24 h in the absence (**a**) or the presence of liposomes (**b**) or ApoBLf (250 µg/ml), either free (**c**) or entrapped into liposomes (**d**, **e**). At the end of incubation time, cells were fixed in methanol, stained with Hemalaum-Eosin and finally examined by light microscopy. Perinuclear protrusions and small apoptotic bodies are observed (**e**)

accumulation, B16-F10 cells were pulsed with equal amounts of free or liposome-entrapped ApoBLf for 30 min, washed and chased for 0.5, 1 and 24 h at 37°C with liposome-free medium. As shown in Fig. 4a, a fluorescent signal could be detected on cells chased 0.5 h after treatment with ApoBLf. The

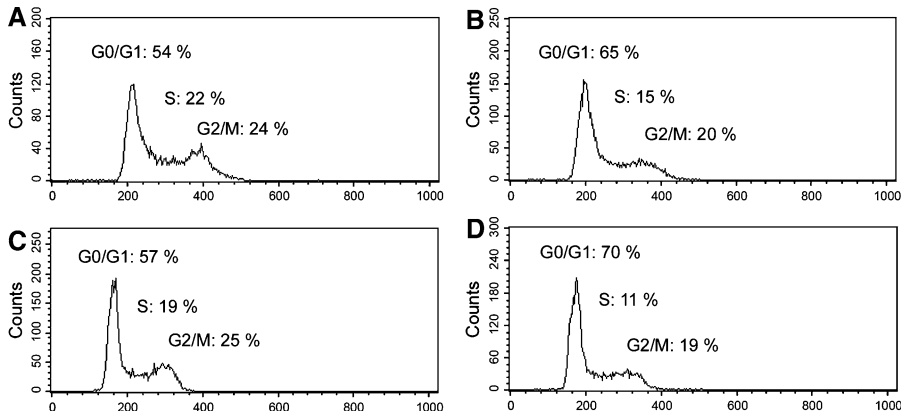


Fig. 3 FACS analysis of cell cycle B16-F10 cells, untreated (**a**) and treated for 24 h with 250 μg/ml free ApoBLf (**b**), empty liposomes (**c**), or lipo-ApoBLf (**d**) were stained with propidium iodide and then analyzed by flow cytometry. *Numbers* indicate the percentage of cells in the different phases of the cell cycle (G0/G1; S; G2/M)

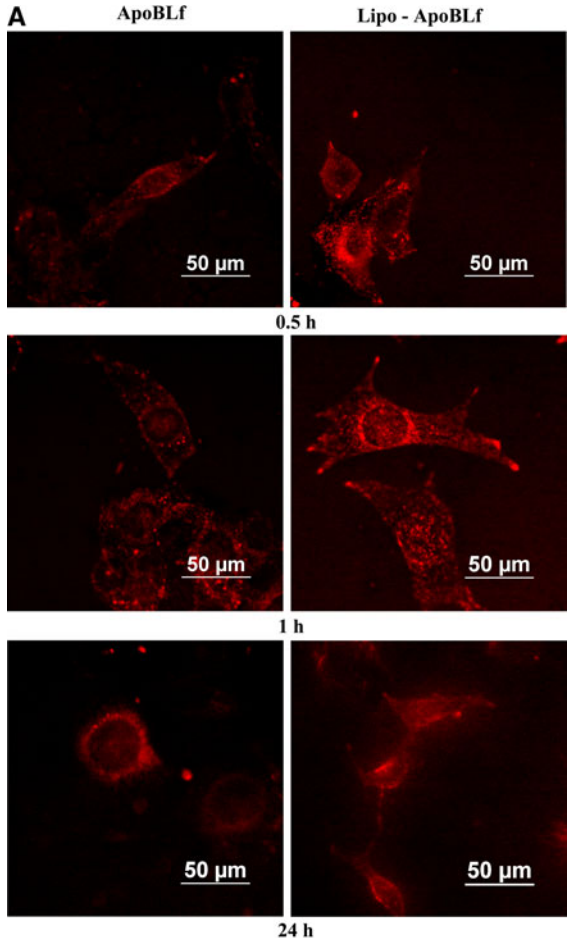
intensity of the fluorescence increased at 1 h and the signal could be still detected on cells chased 24 h, but at lower intensity. It seems that ApoBLf entered the melanoma cells and localized in small vesicles distributed uniformly into the cells cytoplasm. Experiments performed with lipo-ApoBLf resulted in an increased fluorescent signal compared to free protein, irrespective of the chase time. As for ApoBLf, after 24 h the cell fluorescence decreased, probably due to the recycling of the released protein to the cell surface. Compared to ApoBLf, Lipo-ApoBLf complexes showed an accumulation in the perinuclear and dendrites regions. This localization will be further investigated more in depth in order to explain the involvement of liposomes in lactoferrin uptake and traffic.

The increase in cellular fluorescence when cells were incubated with lipo-ApoBLf is the results of the enhanced uptake of protein in its liposomal formulation, as revealed by flow cytometry. FACS measurements of the mean fluorescence intensity, an indicator of internalization, demonstrated that cell fluorescence was increased by 15 to 44% in lipo-ApoBLf treated cells compared to free protein, depending on the incubation time. Although at 24 h the signal decreases, the amount of BLf inside the cells is higher when the protein is entrapped in liposome complexes.

The regulation of cell growth by Lf has been well documented (Ward et al. 2005; Legrand et al. 2008; Jeffrey et al. 2009), and such activity involves binding and internalization of Lf followed by

Fig. 4 Uptake of ApoBLf and lipo-ApoBLf in B16-F10 cells. ▶ **a** B16-F10 cells were treated 30 min at 37°C with 50 μg/ml free or liposome-entrapped Texas Red labeled ApoBLf and after washing incubated in for 0.5, 1 and 24 h at 37°C. Live cells were analyzed by fluorescence microscopy (photos 40×). All the images shown are representative of at least two independent experiments. **b** B16-F10 cells treated with ApoBLf, either free or liposome-entrapped at a final concentration of 1 mg/ml were incubated 0.5, 1 and 24 h at 37°C. Then cells were treated with fixation/permeabilization kit (see Materials and methods), incubated with goat anti-bovine Lf antibody followed by Alexa Flour 488 donkey anti-goat IgG and finally subjected to FACS analysis. The mean fluorescence intensity (GeoMean) was determined using CellQuest Pro software (BD Biosciences). Statistical data were represented from three independent experiments

activation of different signaling pathways. It has been reported that the molecular mechanism of the antiproliferative effect of Lf in Jurkat leukemia T cells is mediated through the modulation of the JNK-associated Bcl-2 signaling pathway (Lee et al. 2009). In the case of drugs included into liposomes, the intracellular delivery of the content is a complex process, which depends upon the surface charge and size of liposomes, and the type of cells (Trif et al. 2001; Mustata et al. 2009). Generally, cellular uptake of liposomes is mediated by their absorbtion on the cell surface and subsequent endocytosis. In this case liposomes can be either delivered by endosomes into lysosomes or en route to lysosomes they provoke endosome destabilization resulting in drug release into cytoplasm. The lysosomal compartment is aggressive against sensitive drugs such as the protein, Lf. The fact that in our study the liposome



formulation of ApoBLf led to an increased accumulation of protein into cells suggest that positively charged liposomes overcome this problem and could be used as efficient delivery system of Lf.

In conclusion, according to the in vitro experimental model used in this study encapsulation of ApoBLf into positively charged liposomes enhanced its inhibitory effect on murine melanoma cell growth and cell cycle evolution. Based on the results of fluorescence microscopy and flow cytometry, the effect appeared to be due to the capacity of liposomes to increase the uptake of the protein by the cells and probably to protect its degradation. In this regard it will be interesting to follow the intracellular trafficking of free and liposome-entrapped Lf in comparative studies and therefore to gain further insight into the molecular and cellular mechanism of antitumoral action.

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