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Original article

Cationic lipid:DNA complexes allow bleomycin uptake by melanoma cells

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ABSTRACT

Bleomycin is a chemotherapeutic agent barely diffusible through the plasmatic membrane. We evaluated DNA/cationic lipids complexes (lipoplexes) as mediators of its uptake in four spontaneous canine melanoma derived cell lines (Ak, Bk, Br and Rkb). Cell survival after lipofection plus or minus bleomycin was determined by the acid phosphatase method and the cellular uptake of lipoplexes, carrying the *E. coli* β-galactosidase gene, was evidenced by SYBR Green I staining. The four cell lines resulted sensitive to the bleomycin/lipoplexes system in both spatial configurations. Survival rates values were lower than 20% in monolayers of the four tested lines and lower than 30% in three lines (Ak, Bk and Rkb) when grown as spheroids. The sensitization to bleomycin depended on lipoplexes in Ak and Rkb while Bk (in both spatial configurations) and Br (as monolayers) were sensitive to bleomycin alone. Although some degree of sensitivity to bleomycin was induced by cationic lipids alone in Ak and Rkb monolayers, the maximal bleomycin effects appeared in the presence of lipoplexes. The sensitization was independent of transcriptional activity. The co-administration of lipoplexes diminished bleomycin IC_{50}: 10-fold in Ak and Rkb monolayers; and sensitized the Ak and Rkb resistant spheroids. The bleomycin cytotoxic effects depended on lipoplexes concentration and diminished when cells were incubated at 8 °C. Our results suggest that lipoplexes sensitize cells to bleomycin, increasing its uptake by an active transport mechanism, such as endocytosis. The bleomycin/lipoplexes system appears as a promising combination of chemotherapy and non-viral cancer gene therapy.

1. Introduction

Bleomycin (BLM) is a glycopeptide antibiotic with antineoplastic activity due to its endonuclease activity [1]. It has been proposed as a chemotherapeutic agent for melanoma since the skin cells do not express a hydrolase capable of inactivating the drug [1]. The cytotoxicity of BLM to certain tumor types might be related to the efficiency of drug uptake. However, BLMs are large (molecular weight of 1.5 kDa), hydrophilic peptides, barely capable of diffusing through the plasmatic membrane. Moreover, different strategies have been developed to bypass the cytoplasmic membrane barrier: Bleosome™ [2], Sendai virus envelope conjugated with cationic gelatin (CG-HVJ-E) [3] and cell electroporation [4,5]. The first two strategies deliver the BLM, using vehicles as ultra-deformable liposomes (Bleosome™) [2] and CG-HVJ-E [3]. The third strategy, cell electroporation, sensitizes the cells to the cytotoxic effects of BLM by increasing the amount of BLM molecules in the cytoplasm [4,5]. This approach was successfully applied to dog patients as monotherapy for melanoma [6] or combined with immunogene therapy for recurrent squamous cell carcinoma [7] as well as for human melanoma patients [8].

Canine malignant melanoma is a spontaneous tumor displaying histopathological features and biological behavior similar to human melanoma, but with a faster progression and an extremely poor prognosis. This highly aggressive canine tumor is too invasive to be cured only by surgical resection and is frequently resistant to current therapies [9,10]. This has prompted investigations to define new treatment strategies.

Here, we are presenting data indicating that cationic lipids, especially when complexed to DNA (lipoplexes, LPX), can efficiently facilitate the delivery of BLM into melanoma tumor cells. Therefore, the co-administration of BLM and LPX would be a viable strategy to enhance the BLM effects by enhancing the BLM assimilation inside the cell via endocytosis.

2. Materials and methods

2.1. Cell cultures

Cultured cells derived from four surgically excised oral (Br, Bk, Rkb) and ocular (Ak) canine melanomas [11] were obtained by enzymatic digestion of tumor fragments with 0.01% Pronase (Sigma, St. Louis, MO) and 0.035% DNase (Sigma) or by mechanical

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disruption in serum free culture medium [12]. They were cultured as monolayers and spheroids at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 with DMEM/F12 medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen), 10 mM HEPES (pH 7.4) and antibiotics. Serial passages were done by trypsinization (0.25% trypsin and 0.02% EDTA in PBS) of sub-confluent monolayers.

2.2. Plasmids

Plasmid psCMVβ carrying the *E. coli* β-galactosidase gene [12] was amplified in *E. coli* DH5α (Invitrogen), grown in LB medium containing 100 μg/ml neomycin and purified using an ion-exchange chromatographic method (Qiagen, Valencia, CA).

2.3. Liposome preparation and in vitro lipofection

DC-Chol (3β[N-(N′,N′-dimethylaminoethane)-carbamoyl cholesterol) and DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethilammonium bromide) were synthesized and kindly provided by BioSidus (Buenos Aires, Argentina). DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphatidyl ethanolamine) was purchased from Sigma. Liposomes were prepared at lipid/co-lipid molar ratios of 3:2 (DC-Chol:DOPE) or 1:1 (DMRIE:DOPE) by sonication as described [13,14]. Optimal DNA:lipid ratios and lipid mixtures were determined for every cell line [12].

Cultured cells at a 5×10^4 cells/cm^2 density (about 40% confluence) were exposed to LPX (0.5 μg plasmid DNA or polydIdC/cm^2) during 3–5 h in culture media without serum. PolydIdC was purchased from Sigma.

2.4. Sensitivity assays

Twenty-four hours after transfection, the βgal-transiently expressing cells were seeded on 96-wells plates as monolayers (4×10^3 cells per well) or on top of 1.5% solidified agar to form spheroids (Ak: 0.5×10^3 cells per well; Bk, Br and Rkb: 5×10^3 cells per well). Unless otherwise indicated, BLM (kindly provided by Gador S.A. Buenos Aires, Argentina) was added to a 3 μg/ml final concentration immediately after the corresponding LPX were added. After 5 days as monolayers or after 12 days as spheroids, cell viability was quantified with acid phosphatase assay (APH) [15]. The cell survival rate was calculated as the ratio of the absorbances between the cells treated and the control cells without BLM.

2.5. Lipoplexes curve and lipofection at low temperature

Dilutions were made from the 1X β-gal LPX described above for dose-response studies. Cells were incubated at 8 °C from 1 hour before lipofection in a box equilibrated with 5% CO_2 inside a refrigerator. LPX and BLM were removed by three washes (5 min each) with cold or pre-warmed DMEM-F12, containing 10% FBS. After 5 days, cell viability of monolayers was determined by the APH assay as described above.

2.6. SYBR green staining of lipoplexes

Plasmid DNA was incubated for 5 min with a 9X concentration of SYBR® Green I Nucleic Acid Gel Stain (Invitrogen) before the DNA/cationic lipids complex formation. Then, lipofection was carried out as described above. Optimal conditions for counting were found at 120 min of incubation. Once finished, cells were rinsed with culture media and fixed sequentially with a solution of 0.05% glutaraldehyde and 1% formaldehyde in PBS.

Photographs were taken using the inverted fluorescent microscope Eclipse TE2000-S (Nikon, Japan) at 200X. The percentage of cells that incorporated LPX was determined by counting the cells with at least one LPX dot over the total of the cells in the visual field photographed. Three different photograph fields were taken per experiment; each experiment was repeated three times.

2.7. K^+ depletion

Potassium was removed as reported in [16,17]. A K^+-free buffer (140 mM NaCl, 20 mM Hepes pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mg/ml D-glucose) was used. Cells were incubated for 5 min with hypotonic buffer (K^+-free buffer/H_2O 1:1) and then washed three times for 5 min with K^+-free buffer. Control cells were subjected to the same treatment in the presence of 10 mM KCl. Finally, in a minimum volume of buffer (with or without K^+), LPX and BLM were added and were incubated for 30 or 60 min and cell survival at day 5 were determined by the APH method as described above.

2.8. Statistics

Results were expressed as mean ± standard error of the mean (SEM) (*n*: number of experiments corresponding to independent assays). Differences between groups were determined by two-way analysis of variance (ANOVA). The difference between means was determined with the Bonferroni post-test.

3. Results

3.1. The presence of lipoplexes overcame the resistance to bleomycin

We assayed the cytotoxic effects of BLM on four canine melanoma cell lines (Ak, Bk, Br and Rkb) previously obtained and characterized in our laboratory [12]. The assay was performed in both spatial configurations: as standard bi-dimensional monolayers and as tri-dimensional spheroids. Ak and Br mono-spheroids were very compact with a spherical symmetry (Fig. 1a) whereas Bk and Rkb spheroids were looser: Bk displaying a big central nucleus surrounded by a close cloud of cells and Rkb a small nucleus surrounded by a large cloud of cells (Fig. 1b).

We tested the sensitivity to the chemotherapeutic agent BLM when it was added to the culture media immediately after the addition of LPX. We chose the pharmacologically relevant BLM concentration (3 μg/ml) reported in previous works [18,19].

As displayed on Fig. 1a,b while Ak and Rkb were not sensitive to BLM alone in any spatial configuration, Bk was sensitive in both configurations and Br only when cultured as monolayers. Lipofection with the reporter gene β-galactosidase (βgal) rendered Ak cells sensitive to BLM (about 80% of cells dying in both spatial configurations). While no significant changes were observed for sensitive Bk. In the case of Br and Rkb, βgal lipofection increased BLM cytotoxicity both in monolayers and spheroids. Compared to their respective monolayers, Br spheroids were relatively less sensitive to BLM, displaying a multicellular resistance (MCR) phenotype. Microscopic monitoring of treated spheroids (Fig. 1a,b) paralleled to the results obtained by the APH assay (Materials and methods). The degree of spheroids compactness did not appear to be related to the resistance to BLM since three out of four spheroids (compact Ak and Br, and loose Rkb) were resistant to BLM alone. Conversely, the four assayed cell lines were sensitive to the drug in the presence of LPX in both spatial configurations.

Rkb monolayers transiently expressing βgal and monolayers and spheroids treated with polydIdC lipoplexes in the absence of BLM, showed significantly lower cells survival when compared to