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Liposome-associated retinoic acid

Increased in vitro antiproliferative effects on neoplastic cells

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The activity of liposome-associated retinoic acid was analyzed on in vitro cultured tumor cell lines and compared to the antiproliferative effects of free retinoic acid. It was found that liposome-associated retinoic acid is about 300 times more active than free retinoic acid in inhibiting in vitro cell growth of leukemic and melanoma cell lines. An increased activity of retinoic acid (10–20 times) was also obtained after premixing of this compound with empty liposomes, demonstrating that the retinoic acid efficiently interacts with liposomes which may facilitate solubility and cell uptake of retinoids.

Retinoic acid; Liposome; Vesicle; Tumor cell growth

1. INTRODUCTION

Retinoids are a class of compounds of great interest, since they are powerful inducers of differentiation in a large variety of experimental systems [1,2] and strong inhibitors of proliferation of normal, as well as neoplastic cells [2]. Among the clinical disadvantages of these compounds is the high toxicity (hypervitaminosis A) and long-lasting teratogenicity [3]. Retinoids bind to an extracellular RSBP and, after transfer to the cytoplasmic compartment of the cells (by a process either spontaneous or mediated by a specific receptor) retinoids bind to a CRABP [4]. This retinoid-CRABP complex is responsible for the activation of a limited set of eukaryotic genes [5].

In the attempt to find alternative ways to administrate retinoids of possible therapeutical interest, we explored the possibility to vehiculate retinoids to tumor cells by the use of liposomes. These vesicles could generally be, indeed, a powerful tool for the vehiculation of drugs to target cells, since vesicles (i) decrease drug toxicity; (ii) exhibit pharmacokinetics and bioavailability sharply different from those of the free drugs and (iii) can be functionalized by coupling either with monoclonal antibodies or other signal molecules (glycopeptides, hormones) leading to cell or tissue-specific releases [6,7]. In addition, liposomes could lead to an improvement of the low water solubility of lipophilic drugs. With respect to retinoids, this latter feature could be of great interest, since the low water solubility is a problem for the administration of these compounds.

Here we show that retinoic acid dramatically increases its antiproliferative effects toward tumor cell lines, when vehiculated with phospholipid vesicles.

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Abbreviations: RA, retinoic acid; MLV-REV, reverse phase evaporated multilamellar vesicles; RSBP, retinol-specific binding protein; CRABP, cellular retinoic acid binding protein; PC, phosphatidylcholine

2. MATERIALS AND METHODS

2.1. *Liposome preparation*

MLV-REV made of egg PC and RA (abbreviated as MLV-REV(RA)) were prepared in a similar way as described elsewhere [8]: 50 mg of egg PC (Lipid Products, Surrey, England) dissolved in 2 ml chloroform-methanol (2:1) and 0.7 mg of retinoic acid (Sigma, St. Louis, MO) in chloroform were dried in a 50 ml round bottom flask (retinoids/lipid molar ratio of 1:25) under nitrogen using a rotatory evaporator. Residual solvent was removed under reduced pressure, and the resulting dried lipid-RA film was then dissolved in 2 ml of diethyl ether. To this solution, 4 ml of isotonic Palitzsch buffer [8] were added, and the buffer-lipid-RA mixture was sonicated at 0°C for 10 min in a Bandelin Sonorex TK52 bath sonicator under nitrogen. The ether present in the emulsion was then removed by rotary evaporation under reduced pressure at room temperature, resulting in a turbid, white liposome dispersion. Separation of liposome-associated RA from free RA was performed by gel filtration using a Sepharose 4B column (Pharmacia, Uppsala, Sweden) (1.5 cm diameter, 50 cm length) [9].

The UV/VIS spectrum of each fraction was measured both in isotonic Palitzsch buffer and in methanol in order to estimate the amount of liposome (from the turbidity at 600 nm) and in order to determine the concentration of RA (from the optical density of clear methanol solutions) at 351 nm, at the absorption maximum of RA. Empty MLV-REV were prepared in a similar way with the exception that the addition of the RA solution was omitted.

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2.2. *Freeze fracture electronmicrographs*

Freeze fracture electronmicrographs were taken by Dr E. Wehrli at the Institute of Cell Biology of ETH, Zurich. The samples were frozen (from room temperature) by the propane-jet technique [10,11], and the cryofixed preparations were fractured at 108K in a Balzen BAF 300. The pressure was 10^{-5} Pa. Platinum/carbon replicas were produced and examined in a Philips EM 301 at 100 kV. Photomicrographs were taken on Agfa Scientia 23D56 cut films and developed in Geratone G5C for 3.5 min at 293K.

2.3. *Determinations of effects on cell growth*

Effects of RA and MLV-REV(RA) were determined on the following in vitro cultured cell lines: the human leukemic K562 [12], the murine erythroleukemia FLC [13] and the human melanoma Colo 38 [14]. Standard conditions for cell growth were α-medium (Gibco, Grand Island, NY), 50 mg/l streptomycin, 300 mg/l penicillin, supplemented with 10–15% fetal calf serum (Flow Laboratories, McLean, VA) in 5% CO_2 at 80% humidity. Cells were usually seeded at the initial densities of 10^5 cells/ml and counted with a Model ZF Coulter Counter (Coulter Electronics, Hialeah, FL).

3. RESULTS AND DISCUSSION

3.1. *Preparation of liposome-associated retinoic acid*

Since retinoids are not water-soluble, we prefer the term 'liposome-associated' instead of the usually used term 'liposome-entrapped'. In fact, it is likely that retinoids are not solubilized into the internal aqueous space(s) of liposomes, but associated (intercalated) within the phospholipid bilayer(s).

The preparation of liposome-associated RA has been performed by the reverse phase evaporation method [15]. The corresponding elution profile of MLV-REV(RA) obtained after Sepharose-4B chromatography is shown in fig.1A. One major peak, containing liposome-associated RA is present in the elution profile. This indicates that a high yield (over 95% of the total RA) of liposome-RA association is reached under these experimental conditions. This high yield is a direct evidence for interactions between RA and the PC molecules, as discussed above. The second and smaller peak in the chromatogram represents the elution of non-associated RA.

The characterization of the MLV-REV(RA) present in the first peak was assessed by electron microscopy (fig.1B). From this analysis, consistent with previously published results [15], it is obvious that the major components (over 85%) of the liposome preparation are large unilamellar vesicles; we, however, prefer to maintain the denomination MLV-REV according to the preparation method.

3.2. *Comparisons of the antiproliferative activity of RA, MLV-REV(RA) and empty MLV-REV*

In order to determine whether liposome-associated RA maintains or even enhances its in vitro antiproliferative activity, K562 erythroleukemic cells were treated with the same amount (in terms of RA) of free or liposome-associated RA. Parallel control cultures of K562 cells were prepared by adding empty MLV-REV,

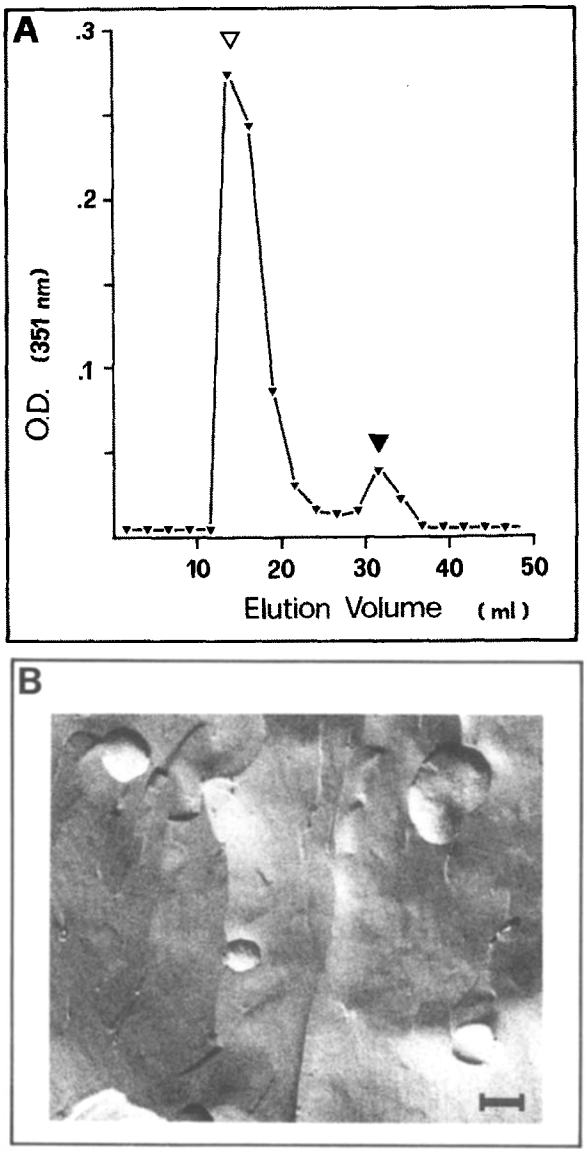

Fig.1. (A) Elution profile of MLV-REV(RA) on Sepharose 4B gel-filtration column (length, 50 cm; diameter, 1.5 cm; flow rate, 160 μl/min, 3.2 ml/fraction). The MLV-REV were prepared by the reverse phase evaporation method (see section 2). The open triangle (▽) indicates void volume fractions, including liposome-associated RA; the solid triangle (▼) indicates fractions containing free RA. (B) Electron microscopy of a MLV-REV(RA) preparation by freeze-fracture analysis. Bar = 100 nm.

at the same phospholipid concentrations used with MLV-REV(RA). After 5 days of cell culture, the cells were counted and the number of cells per milliliter were compared with the values obtained in the case of untreated cells.

Fig.2A indicates that the ID_{50} is about 70 μM for free RA and 0.2 μM for MLV-REV(RA); it is therefore suggested that MLV-REV(RA) exhibit a antiproliferative activity which is about 350 times higher than the activity of free RA. Empty MLV-REV do not cause any inhibition of K562 cell proliferation when used at PC concentrations ranging from 0.05 to 1.6 mg/ml. The higher

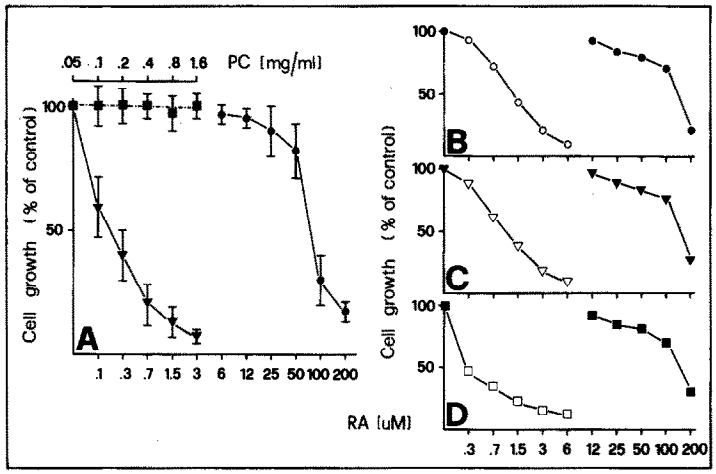

Fig.2. (A) Effects of free RA (●), MLV-REV(RA) (▼) and empty MLV-REV (■) on proliferation of K562 human erythromyeloid cells. Determinations were performed after 6 days of cell culture and represent the means ± SD of 6 independent experiments (the empty and RA-associated vesicles were added at the same indicated concentrations of PC). (B–D) Differential antiproliferative activity of RA (filled symbols) and MLV-REV(RA) (open symbols) on murine erythroleukemic FL cells (B), human melanoma Colo 38 cells (C) and human erythromyeloid K562 cells (D). Determinations were performed after 4 (B) and 6 (C,D) days of cell culture.

activity of MLV-REV(RA) with respect to free RA was consistently reproduced in six independent experiments (fig.2A) on K562 cells and was found to occur by using different tumor cell systems (fig.2B–D).

3.3. *Effects of empty liposomes on RA-mediated inhibition of cell proliferation of K562 cells*

In order to determine whether addition of empty phospholipid vesicles retains effects on RA-mediated antiproliferative activity, increasing concentrations of free RA were premixed with different amounts of empty MLV-REV and the effects on cell growth were determined after 3 days of cell culture. The results obtained are shown in fig.3. When different concentrations (ranging from 6 to 100 μM) of RA are premixed with different amounts of empty liposomes (with PC concentrations ranging from 25 to 200 μg/ml) it is again evident that the association of empty liposomes with RA enhances the antiproliferative effects of RA toward K562 cells.

3.4. *Conclusions*

The main problems in chemotherapy of cancer patients is the toxicity of the drugs employed, the lack of specific vehiculation of drugs and, in some instances, the solubility of drugs in the body fluids [1]. All of these disadvantages are a characteristic of most of the natural and synthetic retinoids proposed for therapy of a wide spectrum of pathologies, including proliferative dermatological pathologies and neoplastic diseases. In this study, we confirm recent published observations [16,17] showing that RA can be efficiently associated to liposomes. In addition, our results demonstrate that liposome-associated RA exhibits an improved in vitro antiproliferative effect compared to free RA.

This increased effect could be due to an increased uptake and/or increased solubility of the retinoids. Accordingly, retinoid transfer across and between phospholipid bilayer membranes has been suggested to occur as a spontaneous and rather rapid process with a half-life of less than 30 s [16]. In addition, retinol transfer between liposomes has also been characterized as a rapid process with a half-life of less than 10 min [16]. These results, when considered together with our data on: (i) the yield of RA-associated liposomes (fig.1A); and (ii) the effects on tumor cell proliferation (figs 2 and 3), suggest that the interactions between liposomes and retinoids are a dynamic process. We suggest therefore that retinoids exhibit a high affinity for phospholipids (fig.3).

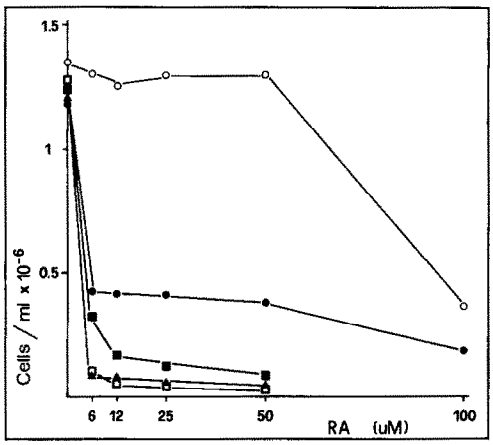

Fig.3. Effects of increasing concentrations of empty liposomes (0.2 (●), 0.4 (■), 0.8 (▲) and 1.6 (□) mg/ml of PC) on the antiproliferative activity of RA. ○, no addition of empty liposomes. Determinations were performed on human erythromyeloid K562 cells after 6 days of cell culture.

In conclusion, our results suggest that liposome-associated retinoids could be proposed: (i) for in vitro studies on the expression of the intracellular RA binding protein; (ii) for the analysis of the effects on gene expression, especially focused on differentiating experimental systems; and (iii) for in vivo treatment of animals after optimization of the liposomes with respect to stability in the blood circulation. The in vivo treatment of tumor-bearing animals is necessary in order to determine the possible applications of liposome-associated RA in experimental anticancer therapy.

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