Research Article

Human erythrocyte-derived nanovesicles can readily be loaded with doxorubicin and act as anticancer agents.

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Abstract

Purpose: In future therapeutics new formulas are needed that assure lower doses, fewer side effects, targeted administration and protection of the drug from degradation. In a first step to fulfil the requirements defined above, we carried out an in vitro study by developing a new procedure to encapsulate drugs using native vesicles first from prostasomes and then from erythrocyte membranes known to be well tolerated. The new method for production of drug delivery vesicles utilized osmotic loading of detergent resistant membranes (DRMs).

Materials and methods: DRMs of prostasomes and prepared human erythrocyte membranes were extracted and separated in a sucrose gradient at a density of 1.10 g/mL containing 1% Triton X-100. These DRMs were characterized by electron microscopy (transmission and scanning EM) and loaded with low and high molecular compounds. PC3 prostate cancer cells were treated with doxorubicin loaded DRMs in triplicate. DAPI (nuclear fluorescent stain) was included and fluorescence microscopic pictures were taken before the cells were trypsinized and counted after 48h.

Results: The content of the well separated band was observed ultrastructurally as small spherical, double layered membrane vesicles, (DRM vesicles) which harbored hyperosmolar sucrose of the gradient. Encapsulated hyperosmolar sucrose induced a transient osmotic lysis of the DRM vesicles when suspended in isotonic buffer containing loading molecules allowing vesicular inclusion. After this proof of concept, the method was finally employed for doxorubicin loading of DRM vesicles from human erythrocytes. When incubating such vesicles with PC3 cells a complete arrest of growth was observed in sharp contrast to PC3 cells incubated with plain doxorubicin in similar conditions.

Conclusion: The present results open up new possibilities for using DRM vesicles as drug delivery vesicles.

Keywords: Prostasomes, Prostate cancer, DRM vesicles, drug delivery

INTRODUCTION

Although systemic chemotherapy is widely used in cancer treatment, effective dosage of the drug is often limited due to serious side effects. Ideally the drug should therefore explicitly be delivered to the

cancer cells. One way of achieving this is to load vesicles derived from biological membranes to form therapeutic vesicles with the drug of choice. Such vesicles have the capacity to include cell specific ligands and they are also protective against

degrading forces from the blood. Accordingly, a given cell may encounter extracellular vesicles (EV) with different origins. Still, there is selectivity in EV uptake as indicated by specific EV-cell interactions conducted by coordination of surface ligands on both EV and the target cell in question (1).

Biological membranes are compartmentalized with surface domains exhibiting various functions at given times. One subclass of these domains is detergent resistant membranes (DRMs), or lipid rafts (rendering them a priori robust properties), which withstand treatment of non-ionic detergents at a low temperature (2). DRMs are thought to play a key role in providing an environment supporting the assembly of surface complexes, e.g. receptor and adhesion complexes (3) and in protein sorting into exosomes (4). Prostatosomes are exosomes secreted from epithelial cells of the prostate (5,6). They are formed in the endosomal compartment by multiple invaginations of the limiting membrane giving rise to intraluminal vesicles (5). The resulting organelle, multivesicular body, fuses with the plasma membrane and releases the intraluminal vesicles by exocytosis as extracellular vesicles (prostatosomes/exosomes) (7). The invagination events are supposed to occur in lipid raft domains in the limiting endosomal membrane (8). DRMs contain lipids with a high ratio of saturated fatty acid chains providing space for cholesterol molecules to intercalate, which in turn yields a highly ordered raft in the less ordered surroundings (9). This was concordant with very high order parameters deduced from electron spin resonance spectra of spin-labeled fatty acids incorporated into prostatosome membranes (10). A feature of prostatosomes and more generally all exosomes is also their high amount of cholesterol and sphingolipids (10,11), again resembling those of lipid raft domains. Lipid rafts have been extracted from prostatosomes and 13% of total membrane (on a protein basis) was estimated to be lipid raft domains (12). A majority of prostatosomes has a diameter in the range of 30-200 nm (13). From here on we will use the more general term "detergent resistant membranes" (DRMs) instead of "lipid rafts".

The aim of the study was in a first step to develop a technique of using DRM domains of exosomes as therapeutic vesicles in an in vitro setting. There are reports on procoagulant activity of not only prostatosomes (14,15), but also exosomes of other origins (16). Therefore, a second step was taken

where extracted DRM domains of erythrocytes, instead, were used for vesicle production. Such vesicles should exhibit low immunogenicity, at least as long as the blood group is compatible with that of the recipients. In the DRM-extraction and separation procedure the original cell structure is dissembled by Triton X-100 during sucrose gradient ultracentrifugation (12), thus creating a new type of nanovesicle mainly consisting of lipid rafts. Furthermore, we introduced a novel loading method of the DRM vesicles. Since the DRM vesicles enclose sucrose with high osmotic strength during vesiculation, a following lysis event during transfer into isotonicity (using the osmotic potential) will transiently open up the formed DRM vesicles and thereby enabling the influx of surrounding drug-containing isotonic buffer. The new type of nanovesicle could be loaded with doxorubicin in this way and be functional in arresting growth of malignant prostate cells in vitro.

MATERIALS AND METHODS

The research was undertaken on deidentified pooled human seminal plasma and donated blood at the blood bank and was approved by the Ethical Committee of Uppsala University (UPS 01-367). Experiments were performed according to the Declaration of Helsinki. Informed consent was obtained from seminal plasma and blood donors at donation.

Purification of prostatosomes

Seminal plasma remaining from male infertility investigations on sperm was collected at the Fertility Clinic of the University Hospital of Uppsala, following fixed routines. Pooled seminal plasma samples were centrifuged for 10 min at 3,000 g and 4°C to pellet possible cells and debris. The supernatant was subjected to another centrifugation for 30 min at 10,000 g and 4°C in order to eliminate possible larger vesicles. The new supernatant was ultracentrifuged for 2 h at 100,000 g and 4°C (using rotor 90 Ti, Beckman Coulter, Brea, CA, USA). The pellet was resuspended in phosphate buffered saline (PBS) and separated on a size exclusion chromatography column (XK16/70 Superdex 200 gel column, GE Healthcare, Uppsala, Sweden) equilibrated with PBS, to exclude amorphous material (17). Fractions were collected at a flow rate of 5 mL/h resulting in fraction

volumes of 1.3 mL. Fractions containing prostasomes were monitored by elevated absorbances at 260 nm and 280 nm (nucleic acid and proteins, respectively). Fractions with elevated absorbances were ultracentrifuged for 2 h at 100,000 g and 4°C to collect prostasomes. The resulting pellet was resuspended in PBS and top loaded on a density gradient of 1M sucrose, 1.5M sucrose and 2M sucrose and ultracentrifuged for 21 h at 85,000 g and 4°C (using rotor SW28.1, Beckman Coulter). The main fraction on top of 1.5M (density range 1.13-1.19 g/mL) was pelleted for 2 h at 100,000 g and 4°C and then resuspended in PBS. The protein concentration was adjusted to 2 mg/mL by a BCA protein assay kit (Merck, Darmstadt, Germany) and kept at -70°C.

DRM extraction from prostasomes

DRMs were extracted according to a previously published method (12). In short, prostasomes (approximately 8 mg) with a density range of 1.13-1.19 g/mL were top loaded on a density gradient of 0.15M sucrose, 0.8M sucrose (density 1.10 g/mL) and 1.5M sucrose, all containing 1% Triton X-100. The density gradient was ultracentrifuged for 28 h at 256,000 g and 4°C (using rotor SW40 Ti, Beckman Coulter). The DRM fraction was collected on top of 1.10 g/mL sucrose and stored at -70°C for at most one week until use.

Purification of erythrocyte ghosts and extraction of DRM

Concentrated erythrocytes were washed twice by 10 times PBS and centrifuged for 13 min at 2,100 g and 4°C. Washed erythrocytes were equally distributed (3-4 mL) into 8 centrifugation tubes (rotor 90 Ti, Beckman Coulter) and lysed by a hypotonic buffer (PBS buffer without saline) thus producing erythrocyte ghosts. Erythrocyte ghosts were repeatedly washed with the hypotonic buffer followed by centrifugation for 15 min at 21,000 g and 4°C, until most of the hemoglobin was cleared. Washed erythrocyte ghosts were then mixed with sucrose solution to a final concentration of 1.5M sucrose that also contained 1% Triton X-100. A density gradient was incorporated in 6 tubes (SW 40 Ti, Beckman Coulter) with the erythrocyte ghosts in 1.5M sucrose containing 1% Triton X-100 at the bottom, a second layer of 1.1M sucrose containing

1% Triton X-100, and a final layer of 0.8M sucrose containing 1% Triton X-100 (1.10 g/mL) on top. Samples were ultracentrifuged for 43 h at 256,000 g and 4°C to obtain DRM. The DRMs were stored at 4°C and were used within 48 h to preserve the asymmetry of phospholipids of the biological membrane.

Transmission electron microscopy (TEM)

Prostasomal DRMs, erythrocyte DRMs and control (unmanipulated) prostates pellets were fixated with PBS containing 2% glutaraldehyde. The fixated pellets were rinsed in PBS followed by post fixation in PBS containing 2% osmium tetroxide for 2 h at 4°C, dehydrated in ethanol followed by acetone and embedded in LX-112 (Ladd, Burlington, VT, USA). Ultrathin sections (approximately 50-60 nm) were cut by a Leica ultracut UCT (Leica, Wien, Austria). Sections were contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 12 Spirit Bio TWIN transmission electron microscope (FEI Company, Eindhoven, The Netherlands) at 100 kV. Digital images were taken by a Veleta camera (Olympus Soft Imaging Solutions, GmbH, Münster, Germany).

Scanning electron microscopy (SEM)

Prostasomal DRMs, erythrocyte DRMs and control (unmanipulated) prostates pellets were fixated with PBS containing 2% glutaraldehyde. The fixated pellets were briefly rinsed in distilled water and dehydrated with 70% ethanol for 10 min, 95% ethanol for 10 min and 99.9% ethanol for 15 min, all steps at 4°C and then transferred to acetone. Pellets were then dried using a critical point dryer (Balzer, CPD 010, Liechtenstein) using carbon dioxide. After drying, the pellets were mounted on separate aluminum stubs and coated with Carbon (Bal-Tec MED 010, Liechtenstein). Analyses were performed in an Ultra 55 field emission scanning electron microscope (Zeiss, Oberkochen, Germany) at 5 kV.

Direct addition to the grid

Aliquots, 5 µL, of the prostasomal DRMs and control prostasomes (2 mg/mL) were placed on two separate grids with supporting carbon films for 5 min. The excess buffer was soaked off by filter paper

and both grids were washed with 10 μ L distilled water for 10 sec. Excess liquid was soaked off and samples were stained with 1% uranyl acetate in water for 10 sec and air-dried. The camera used was Orius SC 200 and analyses of the samples were performed by the software Digital micrograph (Gatan Inc., Pleasanton, CA, USA)

Loading of hemoglobin in vesiculated prostatesomal DRM

The DRM fraction (on top of 1.10 g/mL sucrose) was pelleted for 2 h at 100,000 g and 4°C in PBS. The resulting pellet was suspended in 1 mL tetrameric hemoglobin (European Commission, Reference Material BCR-405) at a concentration of 7.5 mg/mL in PBS, and immediately pelleted by ultracentrifugation for 2 h at 100,000 g and 4°C. The new pellet was washed twice and resuspended in PBS. Absorbance at 415 nm (Soret's band) was measured and a test for tetramethylbenzidine (TMB) peroxidase activity (ELISA setup) was performed.

Loading of gold nanoparticles in vesiculated prostatesomal DRM

The DRM fraction was suspended in 1 mL gold nanoparticles (5-10 nm), 2.5 A520 units/mL in PBS at 4°C (Sigma, Darmstadt, Germany), and then pelleted as stated above for prostatesomal DRM. The pellet was washed with PBS and fixated with 2% glutaraldehyde before TEM analysis was performed.

Western blot

Prostatesomal DRM vesicles loaded with hemoglobin and control prostatesomes (10 μ g each) were separated by SDS-PAGE and transferred to a Novex nitrocellulose membrane, 0.2 μ m (Invitrogen, Carlsbad, CA, USA), for 1 h at 25 V. Unspecific binding sites were blocked by 1% bovine serum albumin in PBS containing 0.1% Tween-20. A primary antibody directed against hemoglobin (chicken anti-Hb, diluted 1:1000, Immunsytem AB, Uppsala, Sweden) was added and was incubated for 1 h at 20°C. A biotinylated rabbit anti-chicken antibody (diluted 1:1000, Invitrogen) was added to the membrane and incubated for 1 h at 20°C. Streptavidin conjugated alkaline phosphatase (diluted 1:1000, Invitrogen) and BCIP/NBT kit (Invitrogen) visualized the

biotinylated antibodies. Analysis was performed on a Molecular Imager ChemiDoc XRS-Imaging System using the software Image Lab 5.1 (Bio-Rad Laboratories, Hercules, CA, USA).

Loading of acridine orange and doxorubicin in human erythrocyte DRM vesicles

Stored (for at the most 48h) human erythrocyte DRMs were evenly divided into 3 ultracentrifuge tubes (rotor 90 Ti) and pelleted in sucrose solutions for 2 h at 100,000 g and 4°C. Pellets in the tubes were resolved in either of 3 one-mL isotonic solutions in order to achieve the post hypertonic lysis event; (i) 1 mg/mL acridine orange (AO, Invitrogen) in PBS, (ii) 1 mg/mL doxorubicin (Actavis AB, Stockholm, Sweden) in PBS, and (iii) PBS only, as control. Formed vesicles were pelleted and washed twice with PBS and ultracentrifuged for 2 h at 100,000 g and 4°C. Each one of the 3 loaded vesicle samples was transferred to a one-mL tube, quickly centrifuged (3,000 g) to pellet unsolved aggregates and the supernatant was transferred to a new tube. Thereafter, vesicles were stored at 4°C in the dark for next uptake experiments with PC3 cells.

Vesicle uptake in PC3 cells

Human prostate cancer cell line PC3 from bone marrow metastasis (prostate grade IV adenocarcinoma), from the American Type Culture Collection (Rockville, MD, USA) (18), was cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin–streptomycin (all from Sigma-Aldrich) in a humidified environment at 37°C, 5% CO₂ atmosphere and routinely tested for mycoplasma contamination.

The cells were trypsinized (0.25% trypsin/ethylenediaminetetraacetic acid (EDTA), Thermo Fisher, Roskilde, Denmark) and 10,000 cells/well were seeded into 8-well chamber slides (Thermo Fisher) and allowed to adhere for 24 h prior to the vesicles uptake experiment. 600 μ L of the AO loaded vesicles were separately diluted in 1.4 mL culture media and 200 μ L of the mix were added to the wells. Cells treated with plain AO received 10 μ g/mL AO. The cells were incubated for; 3, 6, and 16 h (in triplicates) after which the cells were fixated in 3.7% formaldehyde (Sigma-Aldrich) for 10 min at

37°C. The cells were washed twice in PBS and dried for 2 min by treatment in first; 70%, then 85% and finally 99.9% ethanol and kept in the dark at -20°C until analysis.

Vesicle based doxorubicin delivery to PC3 cells

The PC3 cells were trypsinized (0.25% trypsin/EDTA from Thermo Fisher) and 40,000 cells/well were seeded into a 12 well-plate (Sarstedt, Nümbrecht, Germany) and allowed to adhere for 6 h (the adherence time was set to 6 h, in order to prevent the estimated number of cells from proliferating). Doxorubicin loaded vesicles were diluted 1:5 in culture media and 1 mL of the dilution was added to the cells in triplicate. Unloaded (PBS loaded) vesicles were added to cells in the same way as for the doxorubicin loaded vesicles. Plain doxorubicin (0.1 ng) was added to the culture media in the triplicates showing uptake in PC3 cells without vesicle delivery. DAPI (nuclear fluorescent stain) was included in all 4 settings (medium control, empty erythrocyte DRM, plain doxorubicin, and doxorubicin loaded erythrocyte DRM) and fluorescence microscopic pictures were taken (Zeiss Axio Observer (Carl Zeiss, Oberkochen, Germany) before the cells were trypsinized and counted after 48 h (19).

RESULTS

Extraction of prostasomal and erythrocyte ghost DRMs

Prostasomes with a density range of 1.13-1.19 g/mL displayed a high buoyancy band on top of 1.10 g/mL in the sucrose density gradient containing Triton X-100 (for details, see Materials and Methods). Erythrocyte ghosts mixed with Triton X-100 displayed similarly a high buoyancy band on top of 1.13 g/mL. The slightly lower buoyancy of erythrocyte ghost DRMs may be explained by spectrin membrane-skeleton attached to them (20, 21).

Ultrastructure of DRMs

The globular nature was evident in scanning electron microscopy (SEM) not only for prostasomes, but also for prostasomal and erythrocyte derived DRM vesicles displaying in all three cases similarities with diameters of about 100 nm (Fig 1 a-f). The rounded

structure of prostasomes was similarly visualized in transmission electron microscopy (TEM) (Fig 2 a and b) with a more or less electron dense content confirmatory of previous investigations (22). Prostrasomal and erythrocyte DRM vesicles were likewise vesicular-shaped (Fig 2 c-f) but more electron-lucent, especially expressed in erythrocyte DRM vesicles. The size of both types of unloaded DRM vesicles, although rather similar in SEM tended to be more varied when estimated by TEM. Still they were in the same order of magnitude and agreed fairly well with nanoparticle tracking analyses of seminal and PC3 cell exosomes (23). Based on these observations (high buoyancy in sucrose density gradient and ultrastructural findings) allowed us to anticipate that prostasomal and erythrocyte DRM vesicles were comparable entities justifying a switch between these two types of DRM vesicles for further experiments. Alternative fast EM preparation of samples (prostasomes and their separated DRMs) was executed by direct addition to the grid (without fixation) and MQ-water washing for salt removal. Prostasomes were cup-shaped as expected (most probably due to rapid drying up), but prostasomal derived DRMs displayed horseshoe forms with an apparent opening of the globular membrane structure during post-hypertonic lysis (Fig 3 a-d).

In later stages after post hypertonic lysis (Fig 2 c and d) all vesicles are closed suggesting a transient post-hypertonic opening.

Internalization of hemoglobin in prostasomal DRM vesicles

A loading of tetrameric hemoglobin into prostasomal DRM vesicles was possible as demonstrated by western blot. Fig 4A, middle lane, shows a distinct band at about 14 kDa corresponding to dissociated monomeric hemoglobin and a faint band for the dimeric form, while unloaded prostasomes (control, first lane) showed no bands. The vesicular internalization of hemoglobin was further proven by an increase in absorbance at 415 nm (Soret's band, a distinguishing feature of the protoporphyrin ring of hemoglobin) after loading and washing (not shown in figure). The heme moiety of hemoglobin has a peroxidase activity. A peroxidase assay of the loaded and washed prostasomal DRM vesicles was negative (not shown in figure), indicating that no hemoglobin was present at the outer surface of the DRM vesicles and that the

Fig 1. Electron microscopy (EM) findings, scanning EM.

Scanning EM, Fig 1 (a-f) of: intact prostasomes (a and b), bar sizes 100 and 300 nm, respectively; prostasomal-lipid raft vesicles (LRV/DRM) (c and d), bar sizes 100 and 300 nm, respectively; and erythrocyte (LRV/DRM) (e and f), bar sizes 100 and 1000 nm, respectively.

vesicles were sealed. Accordingly, a barrier function is apparent between internalized tetrameric hemoglobin and constituents of the peroxidase assay being outside the DRM vesicles.

Internalization of gold nanoparticles in prostasomal DRM vesicles

In analogy with prostasomal DRM vesicle loading with hemoglobin another set of prostasomal DRM vesicles were loaded with gold nanoparticles at 4°C and immediately pelleted, and then visualized by TEM (Fig 4B). Gold nanoparticles were clearly seen in accordance with our proposed mechanism for

internalization of different non diffusible molecules into DRM vesicles. It should be mentioned in this context that gold nanoparticles do not penetrate biological membranes (24).

Internalization of AO-loaded DRM vesicles in PC3 cells

Acridine orange (AO) is a dye that intercalates double stranded DNA. AO-loaded erythrocyte DRM vesicles were incubated with PC3 cells in triplicate for uptake experiments. Fig 5 illustrates a clear-cut time-dependent uptake of AO-loaded erythrocyte DRM vesicles into PC3 cells (Fig 5 a-c). A maximal

Fig 2. Electron microscopy (EM) findings, transmission EM.

Transmission EM of intact prosomes (a and b), bar sizes 200 and 1000 nm, respectively; prosomal (LRV/DRM) (c and d), bar sizes 200 and 1000 nm, respectively; and erythrocyte (LRV/DRM) (e and f), bar sizes 200 and 1000 nm, respectively.

uptake was observed after 6 h (Fig 5 b). As a control, plain AO was added to PC3 cells under corresponding incubation conditions showing poor uptake of AO (Fig 5 d-f). This internalization of DRM vesicles into PC3 cells was in line with previous uptake studies using malignant and non-malignant cell derived exosomes (23).

Effect of doxorubicin-loaded DRM vesicles on PC3 cells

Doxorubicin is used in cancer chemotherapy and assumed to intercalate DNA similarly to AO. Fig 6A illustrates total cessation of PC3 cell proliferation after 48h by doxorubicin-loaded erythrocyte DRM vesicles in triplicate, reflecting a cytostatic effect contrary to a weak or no effect by plain administration of the drug under corresponding incubation conditions.

The same outcome was visualized by images of DAPI-stained nuclei of treated PC3 cells with few remaining living cells after administration of

Fig 3. Electron microscopy (EM) findings, direct addition to the grid.

Direct addition to the grid of: intact prostasomes (a and b), bar sizes 200 and 500 nm, respectively; and prostasomal (LRV/DRM) (c and d), bar sizes 200 and 1000 nm, respectively.

erythrocyte DRM vesicle-encapsulated doxorubicin (Fig 6B). It can be seen that administered doxorubicin (plain or encapsulated) resulted in swollen nuclei (c, d). Unloaded DRM vesicles (a, b) had no deleterious effect on the PC3 cell proliferation.

DISCUSSION

Considerable efforts are currently put into the area of using extracellular nanovesicles (e.g. liposomes) for chemotherapy drug delivery. Disappointing outcomes using such vesicles are that they commonly interact with macrophages, other innate immune response molecules and even with serum proteins (25). Therefore, they may be destroyed

before they can deliver the drug (26). We introduce a method for production of a new type of nanovesicle. This involves a sucrose gradient separation step in the production of DRMs from human erythrocyte membranes. The plasma membrane is the only subcellular organelle of the erythrocyte and free of nucleic acid fragments (27). The content of the well separated DRM band in the sucrose gradient ultrastructurally appeared as small vesicles of homogenous size (100 nm in diameter, scanning electron microscopy). The position of the band in the gradient corresponded to 0.8M sucrose, i.e. a strongly hypertonic environment. When washing the contents of the band with an isotonic buffer the erythrocyte DRM vesicles underwent a so called post hypertonic lysis (28), involving a transient opening of the vesicular membrane (see Fig 3 c and

Fig 4. Demonstration of loading in revesiculated prostasomal lipid rafts

A. Internalized hemoglobin visualized by immunoblotting. Control (unloaded prostasomes), in first lane (P), and prostasomal DRM vesicles loaded with hemoglobin in middle lane (R). In right lane is molecular weight marker.

B. Internalized gold nanoparticles in prostasomal DRM vesicles are depicted as black dots (see arrows) inside bilayered vesicle membrane.

d). This phenomenon was exploited to allow a loading into the DRM vesicles of constituents (e.g. doxorubicin) present in the isotonic washing buffer.

We thus packaged the cancer drug, doxorubicin, in erythrocyte DRM vesicles and found a complete inhibition of growth of PC3 cells on incubation. This triplicate series of experiments including total cell count of living cells and number of dying cells may serve as proof of concept. Due to their distinctive composition of phospholipids and cholesterol, yielding the membrane a high molecular ordering (10, 11), these DRM vesicles should be considered stable and capable of being frozen with preserved intactness for later use (29), even though our experiments were performed only on short term frozen samples. If obtained from erythrocytes of the same patient or possibly from erythrocytes belonging to the same blood group, they should

have very low immunogenicity. This holds true, given that the asymmetry of the phospholipids of the DRM was maintained and provided that the DRM vesicles were frozen for at the most 48h (29-31). This means a protection of doxorubicin from being destroyed by the body's own defenses and bringing the entire therapeutic cargo to the tumor. A corollary is that a manifold lower dose of the drug can be given in vivo with the same therapeutic effect on the target in question in comparison with administration of plain doxorubicin. In other words, this opens up the possibility to eventually treat patients with smaller and more accurate doses of powerful chemotherapy drugs which in turn may lead to more effective treatment with fewer and milder side effects. An uptake of the erythrocyte DRM vesicles in PC3 cells was anticipated for very good reasons (23). The erythrocyte DRM vesicles by themselves were non-

Fig 5. Comparative, time dependent uptake of acridine orange (AO) into malignant PC3 cells. Representative experiments illustrating PC3 cells treated with AO loaded erythrocyte DRM vesicles in triplicate (upper row) for 3, 6, and 16h (a-c) and uptake of added plain AO (lower row) for 3, 6, and 16h (d-f).

toxic to the PC3 cells. There are previous reports about drug delivery through extracellular vesicles (32-36) but to our knowledge, this is the first report utilizing DRM vesicles derived from human erythrocytes as therapeutic vesicles. Triplicate experiments were considered sufficient for demonstration of this principle of drug delivery, although the results warrant verification by other research groups.

Hypoxia is an important feature of solid tumors because of an imbalance in the supply and consumption of oxygen by tumor cells (37), which in turn is caused by a disturbed allosteric effect on the glycolytic enzyme phosphofructokinase of cancer cells. Tumor cells produce prostasomes/exosomes under hypoxia that modulate the microenvironment to facilitate tumor angiogenesis and metastasis (38-41). Hence, these extracellular vesicles enhance angiogenesis. The newly formed blood vessels, created under disordered conditions, do not represent a normal vasculature. This means that cancer targeting *in vivo* may be obtained passively

by continuous concentration of drug-encapsulated vesicles in tumor interstitial space due to an enhanced permeability of cancer tissue vessels and a retention effect, a so called tumoritropic accumulation (42). Hence, factors of importance are high vascularity, defective vascular architecture, and impaired lymphatic drainage. The easiness of therapeutic vesicle extravasation to the microenvironment of cancer cells is dependent on the size of the vesicle, ideally being 100-200 nm (43) harmonizing with the size of the erythrocyte DRM vesicles thus improving the vectorial delivery of encapsulated doxorubicin to the cancer tissue. Accordingly, tumor blood vessels are more dynamic and variable in comparison with those of normal tissues. Tumor selective extravasation occurs with macromolecules leading to what has been called “the enhanced permeability and retention (EPR) effect” (42,44). The present *in vitro* study does not include any specific mechanisms by which the erythrocyte DRM vesicle surface may be modified to facilitate targeted uptake by tumor cells, while sparing normal cells. The acid microenvironment of

cancer cells favors exosomal fusion with the plasma membrane of cancer cells (39,45,46). Indeed exosomal uptake into cancer cells was approximately 30 times higher than that of synthetic nanovesicles (47).

Drug resistance is a multifaceted problem. The present study suggests that incubation of PC3 tumor cells with erythrocyte DRM vesicles loaded with doxorubicin resulted in a total inhibition of malignant cell growth compared with no inhibition achieved with unloaded vesicles. The intrinsic resistance of cancer cells to chemotherapy drugs is a

conspicuous characteristic of cancer therapy. The microenvironmental acidity with a reversed pH gradient in cancer tissue is an important mechanism of chemoresistance (48). Since many anticancer drugs are weak bases, they are preferentially taken up by non-cancer cells in vivo as such cells are weakly acidic inside and neutral outside. When entering the acidic cancer microenvironment many chemotherapy drugs are protonated and thus neutralized. Doxorubicin is an example of a chemotherapy drug, whose uptake into cancer cells is retarded due to a protonation of the molecule

prior to passage through the cancer cell plasma membrane (46). The DRM vesicles may thus protect doxorubicin from protonation and the vesicle can more effectively internalize the drug. Our findings based on in vitro experiments warrants further in vivo confirmation.

CONCLUSION

We present a new line of action to produce a drug delivery vesicle that can efficiently be loaded even with big molecules with the potential of high bioavailability and biocompatibility. Because of their ideal native structure and characteristics human erythrocyte DRM vesicles are promising drug vesicles for future clinical use.

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Abbreviations:

AO, acridine orange;
DAPI, 4',6-diamidino-2-phenylindole;
DRM, detergent-resistant membrane;
EDTA, ethylenediaminetetraacetic acid;
EM, electron microscopy;
EPR, enhanced permeability and retention effect;
EV, extracellular vesicles;
FBS, fetal bovine serum;
Hb, hemoglobin;
LRV, lipid raft vesicles;
PBS, phosphate buffered saline;
SDS-PAGE, sodium dodecanylsulfate polyacrylamide electrophoresis;
SEM, scanning electron microscopy;
TEM, transmission electron microscopy;
TMB, tetramethylbenzidine

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