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A New Biological Strategy for Drug Delivery: Eucaryotic Cell-Derived Nanovesicles

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ABSTRACT

An efficient drug delivery is the prerequisite of the successful chemotherapeutic treatments of many human diseases. Despite a great number of approaches, the improvement of drug cell internalization remains an actual research challenge. We propose a new biological delivery system based on the extracellular vesicles released by a non-pathological eukaryotic microorganism, Dictyostelium discoideum. After a summary of the main characteristics of these extracellular vesicles, including of their lipid bilayer that appears as a good candidate for initiating membrane fusion, followed by delivery of their encapsulated drug, the capacity of these vesicles to convey drugs into human cells was demonstrated in vitro on two tumor cell lines, resistant leukaemia K562r and cervix carcinoma HeLa cells. A comparison with other extracellular vesicles, like exosomes or bacteria-derived particles, stresses the unique properties of Dictyostelium extracellular nanovesicles for drug delivery.

Keywords*: *Biological Extracellular Vesicles*, *Antitumoral Drug Delivery*, *Dictyostelium discoideum

1. Introduction

The cell is the factory of most primitive unicellular organisms, the building block of plants and animals, as well as of human tissues. In higher eukaryotic organisms, cells are protected by their membrane, from xenobiotics, including many therapeutic drugs. Life dysfunctions, involved in human diseases and cancers, are often to be corrected inside the cell, at different vital targets, like nucleus, mitochondria, Golgi…. Both the old therapies, like antitumoral chemotherapies, and the new ones, like gene therapies, are faced with the major problem of efficient drug delivery. Therefore, the goal of all the drug delivery strategies will be to design Trojan horses to pass the lipid membrane barrier without destroying it and safely convey therapeutic drugs to their cellular targets [1].

Many different Trojan horse strategies for drug delivery have already been designed [2], like using the viral capsides and engineered lipid-like or polymeric vesicles, as well as many new associated technologies [2-4].

A search for the mechanisms, elicited by the non-pathogenic unicellular eukaryotic microorganism *Dictyostelium discoideum* to get rid of many structurally unrelated xenobiotics lead us to elaborate a different biological Trojan horse strategy.

First, we found that these cells "made the difference" between benzo(a)pyrene, B(a)P, the main carcinogenic compound of tobacco smoke, and its non-carcinogenic isomer benzo(e)pyrene, B(e)P. Namely, B(a)P was ejected from the cells, whereas B(e)P could remain into the cells [5]. In the early 1990s, when antitumoral multidrug resistance was thought to be mainly mediated by the P-glycoprotein ABC-transporter, we wondered whether the P-glycoprotein might mediate the B(a)P efflux from the *D. discoideum* cells. Indeed, we evidenced the presence of this human-like transporter in *D. discoideum* cells [6]. However, it was shown to be inefficient for their drug detoxification.

In quite different cell synchronization experiments, we noticed that the widely-used vital DNA-specific stain, Hoechst 33342 (HO342) was unable to stain the nuclei of *D. discoideum* cells. When grown in the presence of HO342, the cell media harboured plenty of fluorescent microstructures, which turned out to be microvesicles, in holding the dye [7].

After studying their characteristics, we discovered that these biologically elaborated nanovesicles, ejected in the extracellular medium, were good candidates for "tricking" the known difficult entry of therapeutic drugs into

human cells. Using HO342, we have shown that the isolated dye-containing vesicles were able to deliver it into the nuclei of naive *Dictyostelium* cells, thus overcoming their constitutive resistance to the free dye. Moreover, with living human leukemia multidrug resistant cells, K562r, known to be resistant to HO342, a *Dictyostelium* vesicle-mediated dye-transfer into the nuclei was also evidenced [8]. Control experiments showed that *Dictyostelium* vesicle-release was not a mere detoxification mechanism, but was both constitutive (Tatischeff, *et al.*, 1998) and important for intercellular communication.

With the goal of using these biological extracellular nanovesicles for drug delivery, an *in vitro* investigation was conducted on HeLa cells, with hypericin, a hydrophobic fluorescent photosensitizer, aimed for antitumoral photodynamic therapy. Thus, we found that *Dictyostelium* nanovesicles, biologically loaded with this therapeutic molecule, are a promising nanodevice for cellular drug delivery [9].

In the present paper, we intend to further propose the use of *D. discoideum* extracellular nanovesicles for drug delivery[1]. We summarize their main characteristics, and stress their efficiency for drug delivery into tumoral human cells, as shown by our *in vitro* experiments. The advantages upon the use of these nanovesicles, when compared with other cell-derived microvesicles are discussed.

2. Materials and Methods

All the materials and methods used in our work on *Dictyostelium* nanovesicles have been detailed in our previous papers [7-9]. Only the methods dealing with *Dictyostelium* cell cultures and preparation of vesicles will be recalled here for clarity.

2.1. Cell Culture

Dictyostelium cells, Ax-2 strain, were grown in suspension in the dark, on a gyratory shaker (150 rpm) at +22˚C, in HL5 semi-defined medium [10], containing penicillin (50 U/mL) and streptomycin (50 U/mL) (Biomedia, Boussens, France). In contrast with mammalian cells, *Dictyostelium* cells are grown without fetal calf serum. For proper oxygenation, each suspension was grown in an Erlenmeyer containing five times the suspension volume.

2.2. Preparation of *Dictyostelium* Nanovesicles

Dictyostelium cell cultures, were initiated at 5×10^5 - 10^6 cells·ml^{-1} and grown in suspension in HL5 medium in the absence (control) or in the presence of the drug to be vectorized. For preparation of nanovesicles, cells were generally used in the late exponential or early stationary phase of growth, at a cell density about 10^7 cells·ml^{-1}. One has to check that the drug does not affect cell growth. After 48 h of growth, both cell suspensions were centrifuged at 700 x *g* for 5 min (+20˚C). The cell-free media were centrifuged at 2000 x *g* for 10 min (+20˚C). The 2000 x *g* supernatants were centrifuged at 12,000 x *g* for 30 min (+4˚C). Nanovesicles present in the pellets were concentrated (×100) in phosphate buffered saline (PBS), pH 7.2 without calcium and magnesium (GIBCO).

These nanovesicles were quite stable in PBS, as they did withstand repeated liquid nitrogen freeze-thaw cycles without breaking. As observed with nanovesicles in holding HO342, they could be kept at +4˚C at least 2 months without releasing the dye.

3. Main Results

In the Eukarya branch of the evolutionary tree, *Dictyostelium discoideum* (http://www.dictybase.org), an ancestral non-pathogenic amoeba, placed at the border between the plant and animal kingdoms, is equipped with a lipid membrane that plays a critical role in many aspects of cell development. As in other eukaryotic cells, *Dictyostelium* plasma membrane contains proteins, lipids and carbohydrates. The phospholipid content of the plasma membrane of axenically grown vegetative Ax-2 cells is not very different from the one of mammalian cell membranes, except for an extremely high amount of unsaturated fatty acids, making up 75% - 90% of the fatty acids of the organism and of the membrane, respectively. The membrane sterol is stigmasterol, instead of the mammalian cholesterol.

3.1. Characteristics of *Dictyostelium* Nanovesicles

Morphological analysis of control vesicles prepared from the cell growth medium was carried out by cryoelectron microscopy imaging. The vesicles appeared mostly smooth and rounded, delineated by a lipid membrane bilayer (**Figure 1**). The histogram of their heterogeneous size distribution indicated that almost 80% of them have an average diameter within a range of 50 - 150 nm [9]. CdSe/CdZnS quantum dots phospholipid micelles were used, to check whether *Dictyostelium* cells might be detoxified from these quantum dots by following the vesicle-mediated pathway. As shown on **Figure 1**, contrary to many xenobiotics, these quantum dots were excreted from *Dictyostelium* cells, as aggregates outside the vesicles. This observation indicates that all the foreign compounds, incubated with *Dictyostelium* cells, are not deto-

[1]Tatischeff, I., Alfsen, A., Lavialle, F. "Extracellular vesicles from non-pathogenic amoebae useful as vehicle for transferring a molecule of interest to an eukaryotic cell" Patent european priority N 03 291 752 07/15/2003 (DRITT-UPMC) (European Patents in Danemark, Deutschland, France, Great Britain, Italy, Netherland and Spain), US Patent and Pending Canadian Patent).

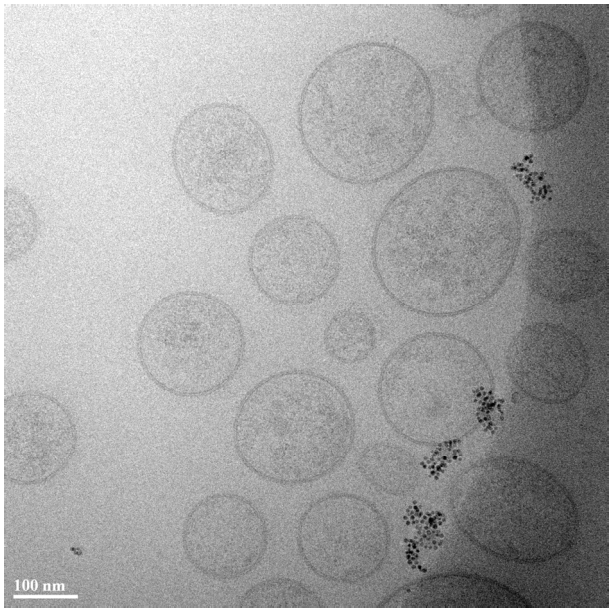

Figure 1. Morphological characterization of *Dictyostelium* nanovesicles by cryoelectron microscopy. For methodological details, cf. [9]. As shown on the figure, CdSe/CdZnS quantum dots phospholipid micelles, incubated with growing *Dictyostelium* cells, were excreted as (black) aggregates outside the nanovesicles.

xified by means of the extravesicular pathway. Further ongoing studies should help understanding the structural characteristics required for a drug vesicle-mediated transport.

A proteomic analysis of the control vesicles revealed a predominance of actin and actin-related proteins. The detection of a lysosomal membrane protein (LIMP II) [9] indicated that these vesicles are likely generated in the late endosomal compartment, as the known exosomes of many mammalian cells.

Lipid analysis of the bilayer surrounding the vesicles [7] showed, beside the phospholipids and lipids common in the plasma membrane: PC, PI, PS and PE, sphingomyelin, PG and DPG, the presence of lyso *bis*-phosphatidic acid (LBPA), a lipid inducing membrane fusion [11]. Noteworthy, this unusual phospholipid is a major membrane constituent of the internal vesicles of multivesicular bodies [12-14], like the known exosomes of many human cells. These observations strengthen the hypothesis of a multivesicular origin for the nanovesicles released by *Dictyostelium* cells and emphasize the role of the nature of the lipids in the formation and function of *Dictyostelium* vesicles.

3.2. Vesicle-Mediated Transfer of Hoechst 33342 to the Nuclei of Human Leukemia Resistant Cells, K562r

HO342-loaded nanovesicles allowed to label the nuclei of naive *Dictyostelium* cells, thus overcoming their constitutive resistance to the free dye [8]. The ability of these nanovesicles, to transfer HO342 to the nuclei of human leukemia living cells, was investigated by adding the nanovesicles (×50) to multidrug resistant K562r cells (**Figure 2**). Four cells were observed by phase contrast microscopy after about 4 h incubation (**Figures 2(a)**, **(c)**, **(e)** and **(g)**). The efficient transfer of HO342 to the cell nuclei was observed under UV excitation. The simultaneous observation of the same cells with white light and UV excitation (**Figures 2(b)**, **(d)**, **(f)** and **(h)**) clearly showed that the HO342-stained nuclei were inside the K562r cells. Thus, the *Dictyostelium* nanovesicle-mediated HO342 delivery had overcome the induced resistance of K562r cells to the vital staining of their nuclei.

3.3. Vesicle-Mediated Transfer of Hypericin to the Golgi of Human Cervix Carcinoma HeLa Cells

Hypericin transfer to target cells was first visualized using human fibroblasts (HS68) as control cells and with HeLa cells used as a model of tumoral cells. After a 1-h incubation in the presence of hypericin packaged into nanovesicles, a fluorescent signal was detected almost exclusively in the perinuclear area of HeLa cells (**Figure 3**). Under these experimental conditions, neither the cell plasma membrane, nor the nucleus, was labeled by the hydrophobic cargo molecule.

Similar experiments were conducted using free hypericin, *i.e.* not encapsulated into vesicles, for comparison. After only a 30-min incubation, HeLa cells became round-shaped, the typical morphology of dying cells that have lost their adhesion properties (**Figure 3**). This result demonstrates that the nanovesicles vectorize significant amounts of drug, but also prevent the uncontrolled cell

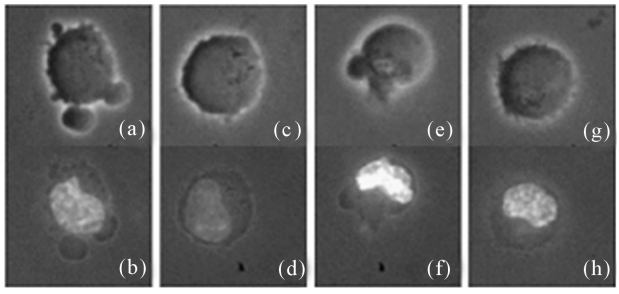

Figure 2. Transfer of HO342 mediated by the *Dictyostelium* nanovesicles to the nuclei of living human leukaemia cells, K562r. Cells were incubated (v/v) with these nanovesicles (×50). Four different K562r cells are shown, in the range of incubation time 3 h 40 - 4 h 10, as observed either with phase contrast (×40 CP objective) (a, c, e, g), or with both white light and UV fluorescence microscopy (×40 UV objective) (b, d, f, h). Figure reproduced with permission from [8].

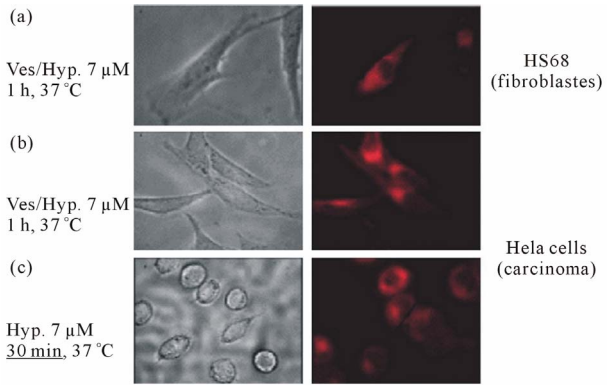

Figure 3. Light-transmission and fluorescence microscopy of hypericin internalization within human target cells. Human fibroblasts (HS68) (a) and HeLa cells (b and c) were incubated at 37˚C in the dark for the indicated times, in the presence of 7 µM hypericin packaged within *Dictyostelium* nanovesicles (a and b) or free in DMEM (c). Figure reproduced with permission from [9].

death triggered by free hypericin under the blue light used for observation.

To check the intracellular localization of hypericin delivered by the nanovesicles into HeLa cells, co-localization studies were performed with fluorescent markers of the Golgi apparatus (BODIPY ceramide) and of lysosomes (LysoTracker). Within 1 h, hypericin was shown to co-accumulate with BODIPY into the Golgi apparatus, and the hypericin-loaded nanovesicles were efficient for photodynamic killing of HeLa cells [9].

The previous studies were initiated to investigate the potential use of *Dictyostelium* cell-released nanovesicles as *in vitro* drug carriers for cancer therapy. *Dictyostelium* cells were able to release into the culture medium nanovesicles loaded either with HO342 or with hypericin, a photosensitizer differing in charge and hydrophobicity from the DNA marker. Additionally, these nanovesicles were shown to respectively label human leukaemic resistant cells K562r, and human cervix carcinoma, HeLa, cells. It is worth stressing that the two cargo molecules, HO342 and hypericin, were transferred to two distinct intracellular locations, namely, the nucleus for HO342 and the Golgi apparatus for hypericin.

Vesicular-mediated delivery of hypericin depicted good photo efficiency for killing HeLa cells. Using vesicular hypericin, instead of free hypericin, ensures a better control of drug cell loading, by an easy manipulation of the amount of vesicles incubated with the cells. Most importantly, the vesicular delivery prevents the detrimental aggregation of free hypericin in aqueous media.

4. Discussion

Cell membranes are a structured self-assembly of lipids interacting with proteins and in contact with both external medium and the internal aqueous solvent and solutes. They constantly form lipid vesicles that either move internally from the cell membrane to sites within the cell or are externalized. These vesicles are formed of a selection of membrane lipids and are characterized by specific properties, either of fusion and/or of targeting, that give them their dynamic properties, allowing the exchange of materials and informations.

From prokaryotic unicellular organisms to primitive eukaryotes, at the border between the vegetal and animal kingdoms, like the amoebae *Dictyostelium discoideum* up to mammalian cells [15] the formation of extracellular vesicles of different sizes, origins and functions, was shown to be a property of almost all cell types. Thus, extracellular vesicles have been described as originating from almost all mammalian cell types: human blood cells (platelets [16], B lymphocytes [15], dendritic cells [17], but also from intestinal epithelial cells [18] or from different pathological cells [15].

All these extracellular vesicles are examples of "vesicles shipping extracellular messages" [19] and "triggering intercellular communication" from one cell to another, either by fusion with the target cell [20] and/or by allowing internalization of signaling molecules [15,21]. These extracellular vesicles are, therefore, also potential Trojan Horses for drug delivery to target cells.

The exosomes, originating from the endosomal traffic [22], are mostly considered as candidates to develop genetic vaccines for immunotherapy [23], but have recently been also considered for drug delivery [24]. However, as mentioned by these authors, many "critical obstacles to the clinical translation of exosome-based therapy" remain. The growth of *Dictyostelium* cells in a semi-defined medium, without any serum, as compared to more complex human cell media, might favor this simple eukaryotic cell model for solving some of the remaining problems. Moreover, it is to be stressed, that our drug delivery device, based on a detoxification process, includes the *Dictyostelium* cell conditioning of the therapeutic drug into the vesicles that will be released. Therefore, no drug internalization agents into the vesicles are needed, as the ones in exosome-based therapy. Bacterially derived particles have also to be further loaded with therapeutic agents, in order to be efficient carriers [25]. Therefore, among all the cell-derived extracellular vesicles, *Dictyostelium* nanovesicles present the unique advantage of being cell-engineered for drug delivery.

With regard to a potential strong immunogenicity of these nanovesicles, due to their microbial origin, which might be a serious drawback for their use *in vivo*, it has already been documented for bacterial derived particles. By contrast, a first *in vivo* study upon the immunogenicity of *Dictyostelium* nanovesicles, intravenously injected

twice in the tail of Balb/C mice, has shown a specific antibody response, but no pyrogenic response nor any inflammation, as measured by five pertinent cytokines (study performed by *Genosafe*, Evry, France). This should minimize the risk of undesirable immune responses upon *in vivo* administration of these biological nanovesicles, such as those triggered by tumor-released exosomes [26].

5. Conclusions

Our work indicates that *D. discoideum* is a bioengineering designer able to formulate vesicular drug carriers. To our knowledge, this is the first study describing cell-engineered vesicles able to both load a therapeutic molecule and vectorize it into human cells, as we have shown with the important antitumoral photosensitizer, hypericin [9].

Other characteristics of *Dictyostelium* vesicles appear interesting for targeting purpose; namely, the presence of discoidins I and II [9], which bind to the cell membrane with a specificity for galactose-related residues, might be of interest for lectin-mediated drug delivery [27]. The targeting strategy, tested with bacterially derived vesicles [25], by using bi-specific antibodies, with one arm recognizing a component of the vesicle surface, the other a cell surface receptor of the target cell, might also be applied to *Dictyostelium* nanovesicles. Another vectorization strategy could be developed by using *Dictyostelium* cells cultured in the presence of magnetic nanoparticles to engineer magnetic nanovesicles [28].

With regard to the possibility of using *Dictyostelium* nanovesicles in therapy, many problems remain to be solved. A study to elucidate the membrane events involved in cell entry of the drug vectorized by the nanovesicles should be performed. For further *in vivo* approaches, toxicity and allergenic properties of more purified nanovesicle preparations are to be characterized. Taking into account the described culture of *Dictyostelium* cells in bioreactors [29], a drug vectorization strategy could, indeed, be developed at a large pharmacological scale.

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