DEVELOPMENT OF THERANOSTIC FERUMOXIDE AND GADOLINIUM NANOLIPOSOMES FOR CONTROLLED RELEASE OF CYTOKINES

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Abstract

Several experimental strategies have been formulated to develop theranostic liposomes by incorporating both drugs and various imaging contrast agents.in last decades. Liposome kinetics and cracking could be potentiated by external forces (ultrasound, electro-magnetic, laser induced). Gadolinium is used in many contrast entities, and they could be detectable by various methods of electron microscopy and tomography if the concentration is within a good range of values. Our workplace is working with a wide range of extracellular vesicles, which are natural transporters of regenerative factors. In order to evaluate the Gd-label effectiveness for some specific extracellular vesicles (specific exosomes derived from mesenchymal stromal cells), experimental labeling methods were prepared. We prepared original conjugation methods for Gd-DSPS binding to the surface lipid layer of extracellular vesicles, and evaluation by electron microscopy and DLS methods was conducted. To reveal possible adverse effects of Gd-vesicles that may be initiated after contact with living cells, we also evaluated basic cytotoxicity tests, and we conducted a statistical survey for metal distribution from dermal dressing into different tissues. Results of tests revealed that vesicles before and after Gd-labeling had very similar sizes (115 versus 122 nm) and similar zeta potential. It has been demonstrated that Gd-vesicles are stable particles in the dermis and subdermal region, still detectable after 12 days as compact nanobodies with high contrast under electron microscopy in dermal tissue. A simple dose of 20 µg per 1 cm² on dermal tissue (mixed in a hydrogel drop) showed very good diffusion into the tissues over the next 6 days. The diffused concentration of vesicles is sufficient for the quantification of particles with mass spectrometry after taking a biopsy, but not yet sufficient for the quantification of particles by traditional X-ray or MRI tomography. Gd-release had no adverse toxicity. A modified variant, “Gd + extra sono-stimul,” was evaluated at the end of the experiment. There is evidence that sono-stimulation could accelerate the penetration of healing vesicles into the target dermal tissue.

Keywords: Nanovesicles, metal toxicity, medical device, tissue model, liposome kinetic

1. INTRODUCTION

Extracellular vesicles (EVs) are microscopical objects released by almost all cell types, sometimes called as nature liposomes, and there are three main groups of EVs according to their size and genesis: (i) apoptotic bodies (>1,000 nm) released during early apoptosis; (ii) microvesicles (100 to 1,000 nm) formed via outward budding of the plasma membrane; and (iii) exosomes (40 to 100 nm) secreted after fusion of multivesicular bodies with the plasma membrane [1,2]. The third group of EVs, exosomes, mostly contains anti-inflammatory and regenerative cargos (cytokines, enzymes and miRNAs) and they have great potential in modern medicine of metabolic disorders and wounds. However, the current understanding of these all nature EVs, especially

their *in vivo* behaviors and distributions, remains inadequate. So, it is necessary to establish marking and detection methods, methods that would be safety for the *in vivo* imaging and tracking of EVs in tissues of animal and human body [3]. Our preliminary result have shown, that labeling by gadolinium could be an optimal and safe way, the initial *in vivo* tests of these methods are based on micromanipulation with EVs lipid bilayer and precise ICP-MS (Inductively coupled plasma mass spectrometry) and MRI detection, and the summary of experiments is presented in this work.

2. METHODS

2.1 Cells and exosomes preparation

The MSCs were isolated from the adipose tissue of three healthy rats, and then expanded in the complete Dulbecco's modified Eagle's medium (5 % FBS) for three weeks, details in [2]. Exosomes were isolated from these adult MSC by combining ultracentrifugation and stimlultaion by AMC generator. The original AMC generator was applied on cell culture 24 hours before ultracentrifugation, generator was designed on our workplace, the aim was to generate sinusoidal induced electric currents with the amplitude modulation and the harmonic carrier frequency of 5,000 Hz and very high current density of induced electrical in cell sample area (above 1 A/m2), details in [4], which accelarate EVs release from cells to the liquid culture medium, ultracentrifugation was used for aseptic separation of exosomes from liuid medium into the 100 sterile microliter vial. Summary of isolation and following Gd-labeling are ilustrated on **Figure 1**. EV with Gd-labels were prepared for in vivo application on dermal tissue (back-side of ZDF rats), EVs were not applied in drop of solution, however mixed with hydrogel Olfen (Teva, Czech Rep.), the amount of EV was adjusted to achieve a final concentration of 20 µg of gadolinium per milliliter of gel.

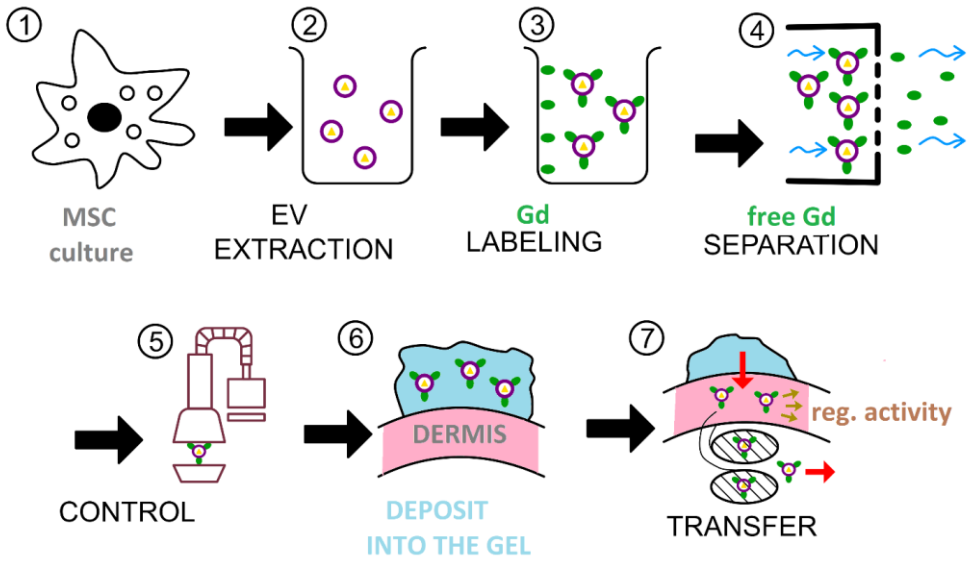

Figure 1 All steps for EV-Gd particles production and application in vivo. Details of step #3,#5 and #7 and their analytical evaluation are described bellow. Step #4 was important for separation of free gadolinium komplex nonincorporated EV, separation separation was realized by a special centrifugation machine ExoDisc (LabSpiner, Korea).

2.2 Exosomes labeling, quantification and transfer

Gadolinium labeled exosome was prepared by lipid insertion following membrane extrusion method [5,6]. In brief, 500 µg DSPE-DOTA-Gd (Avanti Research, USA) were solubilize in 1× DPBS at 65 °C, suspension was cooled down to 25 °C and co-incubated with 500 µg exosome protein for 10 minutes. The exosome and Gd-

complex mixture was sonicated in 1ml plastic vials for 1 minutes (5 W, 10s-ON/10s-OFF, original Sono-Ap machine, by CVUT Prague, Czech Rep.). The mixture was extruded trough 100 nm membrane pore size to unify the hydrodynamic size. All other reagents were purchased from Sigma–Aldrich, if not indicated otherwise.

2.3 Determination of EV-Gd particles before *in vivo* application

EVs were applied to grid coated with Formvar (Sigma-Aldrich, Czech Republic). Philips 208 S Morgagni (FEI, Czech Republic) was used for samples visualization (using 7,500× amplification and a quickening voltage of 80 kV). After that, another part of EVs sample were diluted in Milli-Q water. The diluted suspension was placed in a disposable, low volume cuvette with path length 10 mm (Malvern Instruments, UK). For the liposomes size distribution, the Zetasizer Ultra (Malvern Panalytical, UK) operating at detection angle of 173° at room temperature was used.

2.4 In vivo sampling and determination of Gd content using inductively coupled plasma optical emission spectrometer (ICP–OES) and tissue monitoring

Small samples of gel, samples of dermis or internal organs (all compartements, where the high retention is expected, described in **Figure 1**) was collected in different days after gel application on dermis. Concentration of Gd in these samples was measured by ICP–OES Ultima 2 (Horiba Jobin Yvon, France). The Gd standard for ICP (1 g/l) was used as the calibration solution diluted from 0.001 to 50 mg/l. Measurement was carried out in argon flow and using spectral emission characteristics at wavelength of 342 and 246 nm. Gd deposit was recomputed to whole organ mass.

Clinical tomography machines (1,5 T MRI, Bruker) were prepared for experimental scan of EVs deposit in gel and sub-epidermal layers was tested on at day 1, 6 and 12. At the same time, the analysis of viability of dermal cells was conducted on bioptic sample using histological staining and D/L flow cytometry tests..

3. RESULTS

3.1 In vitro characterization

A typical EV-Gd micrograph is shown in **Figure 2**, the exosome with Gd-labels is compared with unlabeled one. As shown in the figure, the most prominent property of both types of EVs is their almost spherical shape.. Unilamellarity of liposomes and homogenity in the size distribution is in correlation with DLS data, the statistical result for hydrodynamic parameters of unconjugated EV (orang) or Gd-conjugated EV (blue) are 115,5± 8,6 nm 122±12,8 nm.The EVs samples had zeta potential values −16,5±0,6 and −22±0,8 mV, respectively.

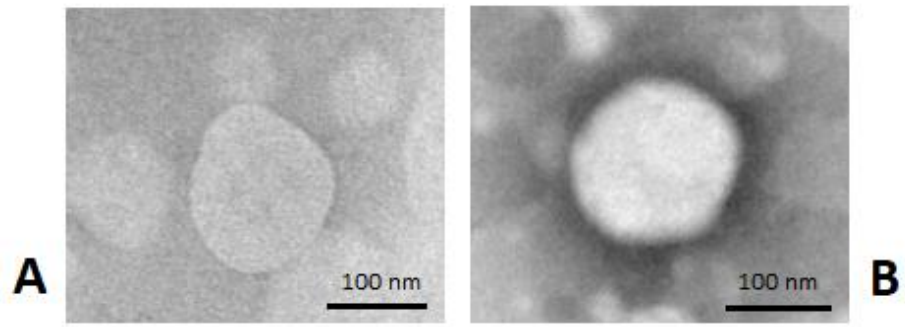

Figure 2 Cryo-TEM images: (**A**) control EV, (**B**) EV after labeling by gadolinium.

3.2 In vivo characterization

The results ICP-MS determination of EV redistribution in different compartment of animal body are summarized on **Figure 4-A**. Very interesting is **Figure 4-B**, which represents modified experimental setup, where the EV

was applied under additinal ultrasound stimuls by Sono-Ap machine (CVUT Prague, Czech Rep.; direct contact of sonotrode, 20 minutes in the morning and in the evening at first day of experiment, animal under anesthesia)

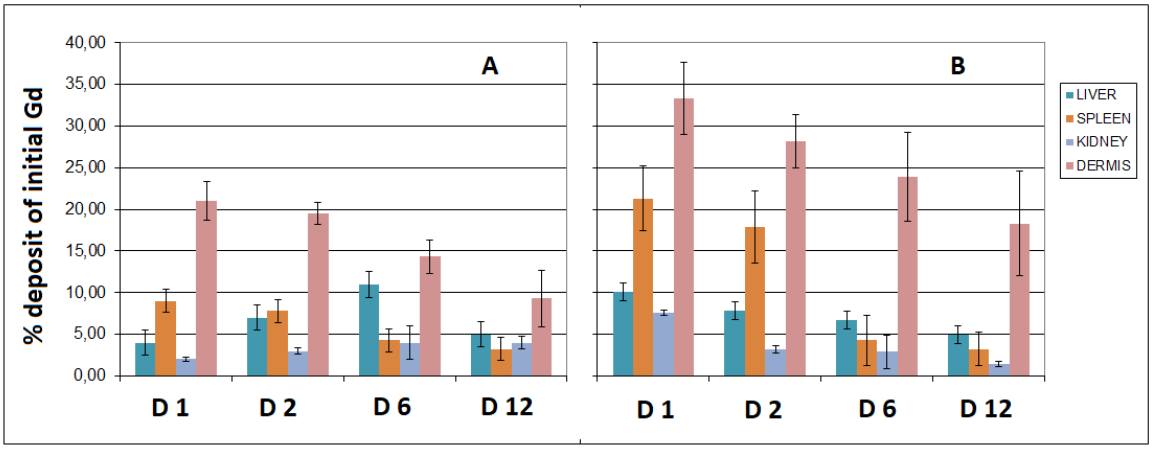

Figure 3 Redistribution of EV-Gd complex and their realtive concentration in different days after initial drop of gel to dermal area. Result derive from ICP-MS determination, absolute mass of Gd for all organs was recomputed as % from initial Gd-cargo of EVs mixed in initial gel drop. Error bars represent the mean standard deviation of 3 samples.

The last experimental step was imaging the gel-drop (20 µg of Gd per 0.5 ml of hydrogel) using an MRI tomography. A concentrated deposit of EV-Gd in the gel can be detected on day 1 (**Figures 4**). In the following days, however, the lower concentrations in the dermis and tissues deeper in the body are not easily detectable in day 6 or day 12 (scan not shown). Improving the contrast of the vesicles and/or the sensitivity of the tomograph will therefore be yet another task for our development in future.

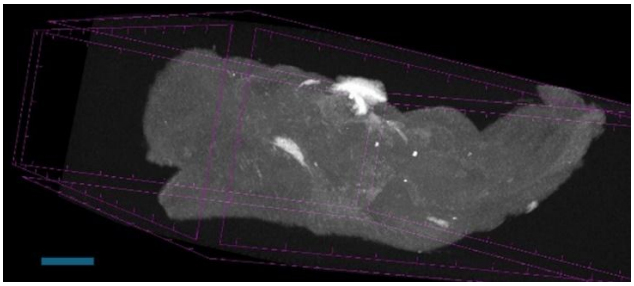

Figure 4 Contrast gel drop containing EV-Gd particles on dermis, scanned by MRI (1.5T). Bar = 1 mm.

Results and their impact can be presented in 4 basic points: (1) The method based on exosomes isolation and Gd-labeling is effective and generates safety particles carrying important pro-regenerative cytokines, no significant effects for viability of cell inside dermis was detected. (2) A gel body was used as a scaffold, from which exosomes diffuse successfully. Unlike intravenous administration of exosomes, the gel body used ensures concentration in the dermal target area, there is evidence from Fig.3 that 20% of initial deposit was transferred to dermis during the first 48 hours; if sono-stimulation is used, this ratio may increase to 33%. (3) Using of the sono-stimulation the longtime concentration of Gd-labels a in internal organs (mainly spleen and liver) can be significantly increased in the first 6 days, however the concentration is relatively well shifting back to under 1 µg/g of tissue after day 12. The clearance of Gd from the the internal organ seems as very effective in all variants of application. (4) The release of cytokines in target dermis was detected by western blot (data not shown), the delivery by used variant of exosomes is effective. Higher local concentration of exosomes and cytokines inside target tissue are a harbinger of a better pharmacological effect.

4. CONCLUSION

EV vesicles have traditionally a problem with the absence of contrast and the possibility of determining the concentration in the target tissue [7,8]. The presented method of gadolinium labeling proved to be effective and safe, it does not significantly change the physical properties of the nature vesicles. Our test of Gd-EVs in vivo approved, that the diffused concentration of vesicles is sufficient for quantification of particles with a mass spectrometry in different dermal and internal location of body (by taking a microbiopsy), but not yet for quantification of particles by traditional X-ray or MRI tomography. And this is a shared unresolved issue in a number of recent publications looking for multimodal high-resolution detection methods [9,10]. We did not detect Gd-induced adverse toxicity. Modified variant “Gd + extra sono-stimul“ was also evaluated, this variant have shown that sono-stimulation could accelerate the penetration of vesicles into the target dermal tissue. Production Gd-labeled vesicles is chance to precise supply and precise monitoring of specific curative agents for variety of dermal pathology.

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