Cancer cell targeting of lipid gene vectors by protein corona

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ABSTRACT

In this study we exposed 1,2-dioleoyl-3-trimethylammonium propane (DOTAP)/DNA lipoplexes to different concentrations of human plasma (HP). Liquid chromatography-tandem mass spectrometry showed that the adsorbed protein corona is rich in vitronectin, a major ligand for $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins. To investigate whether the protein corona could dictate a selective access to cells expressing vitronectin receptors, highly-metastatic MDA-MB-435S cells that express high levels of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, were used as experimental model. Non-metastatic MCF7 cells that do not express $\alpha_v\beta_3$ and express reduced levels of $\alpha_v\beta_5$ integrins were used as a reference. After exposure to HP, the cellular uptake of lipoplexes in MDA-MB-435S cells was more than 2-fold larger than that of bare ones, while complexes could not enter MCF7 cells remaining largely accumulated at the plasma membrane. Collectively our data suggest that the protein corona can be used as a novel tool for cancer cell targeting.

Keywords: protein corona, cationic liposome, DNA, lipoplexes, gene delivery

1 INTRODUCTION

In the past twenty years, the outburst of nanotechnology has revolutionized the field of pharmacology making it possible to deliver a huge variety of biologically active compounds [1]. The most relevant application of nanotechnology in pharmacology is cancer therapy. Over the last decade, a lot of efforts have been devoted in achieving "active targeting" to deliver anticancer drugs to the right cells, based on molecular recognition processes [1]. Several ligands such as monoclonal antibodies, peptides or small molecules targeting proteins expressed on cancer cell membranes or endothelial cells lining tumor blood vessels are among the possible options to perform the active targeting of nanocarriers toward tumoral sites.

Recent landmark studies [2-4] have focused on what cells and organs actually see when interacting with a nanoparticle dispersed in a bodily fluid. It has been proposed that, after exposure to a biological milieu, the unit of interest in the cell-nanomaterial interaction is not the bare nanoparticle, but the complex made of the nanoparticle and its hard corona of associated plasma proteins. Nanoparticles covered by plasma proteins can be internalized by nonspecific interactions. However, since many cellular uptake pathways are indubitably receptor-mediated, a nanoparticle covered by proteins that are not recognized by cell receptors cannot be efficiently engulfed by cells [4]. It is therefore clear that, as direct consequence of the surface coverage of proteins, the active targeting could be compromised. Since nanoparticle must enter the systemic circulation to access its molecular site of action, a question spontaneously arises: Can we use the protein corona as a tool for cancer targeting? To answer this question, a deep knowledge of the protein corona is mandatory.

Among nanotechnologies for cancer treatment, cationic liposome-DNA complexes (lipoplexes) are the most promising non-viral nanocarriers. An accurate knowledge of proteins adsorbed on lipoplex surface is an urgent task for understanding their biological impact. In this work, we investigated the effect of the protein corona on the cancer cell targeting of lipoplexes. To this end, we used 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) cationic liposomes that efficiently deliver DNA in a wide variety of cell lines [5]. First, we assessed a semi-quantitative determination of hard corona composition as a function of increasing human plasma (HP) concentration, combining one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE) and liquid chromatography-tandem mass spectrometry (LC MS/MS) proteomics. The protein corona was found to be extremely rich in vitronectin a major ligand for the vitronectin receptor $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin, which are overexpressed in some tumor cell lines [6]. Thus, we asked whether the adsorbed protein corona could dictate a selective access to cells expressing vitronectin receptor, that are $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Since it has been reported that metastatic MDA-MB-435S cells express high level of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin, we have used this cancer cell line as experimental model. Non-metastatic MCF7 that do not express $\alpha_v\beta_3$ and express minor levels of $\alpha_v\beta_5$ integrins were used as a reference.

The cellular uptake of DOTAP/DNA/HP complexes was investigated by confocal laser scanning microscopy (CLSM) and flow cytometry experiments.

2 MATERIALS AND METHODS

2.1 Chemicals, human plasma and cells

All chemicals were purchased from Sigma Aldrich (St. Luis, MO, USA) unless otherwise stated. The sequencing grade modified trypsin was from Promega (Madison, WI, USA). All organic solvents were the highest grade available from Carlo Erba Reagents (Milan, Italy). Ultrapure water (resistivity 18.2 MΩ cm) was obtained by an Arium water purification system (Sartorius, Florence, Italy). Solid phase extraction (SPE) C18 cartridges were BOND ELUT (Varian, Palo Alto, CA, USA). Sample of human whole blood were obtained by venipuncture of ten healthy volunteers aged 20–40 years. HP was pooled and stored as described elsewhere [7]. For analysis, HP aliquots were thawed at 4 °C and then allowed to warm at room temperature. MDA-MB-435S and MCF-7 cells were purchased from American Type Culture Collection (ATCC) and were grown in Dulbecco's modified medium (DMEM) supplemented with 10% of Fetal Bovine Serum at 37°C and in 5% CO_2.

2.2 Lipoplexes

Cationic DOTAP was purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Calf thymus (CT) DNA was obtained from Sigma-Aldrich (St. Louis, MO) and was used without further purification. Unilamlellar DOTAP CLs (1 mg/mL) were prepared according to standard protocols [5]. Cy3-labeled 2.7 kbp plasmid DNA was purchased from Mirus Bio Corporation (Madison, WI). A solution of CT-DNA (1 mg/mL) was sonicated for 15 min to produce fragments with typical length distributions of between 500 and 1000 base pairs as determined by electrophoresis on agarose gels. For proteomics experiments 100 μL of DOTAP CLs were mixed with 20 μL of CT-DNA. For CLSM experiments self-assembled lipoplexes were obtained by mixing 1 μL of the Cy3-labeled plasmid DNA solution with 5 μL of the DOTAP dispersion. All samples were prepared at a fixed cationic lipid/DNA charge ratio (mol/mol), i.e., ρ = cationic lipid (by mole)/DNA (base) = 2.3. Lipoplexes were incubated with HP at different concentrations (0, 2.5, 5, 10, 20 and 50 % HP) in a saline buffer (pH 7.4).

2.3 PROTEOMICS

After incubation with HP, the samples were centrifuged to pellet the particle/protein complexes. The pellet was resuspended in the saline buffer [7], transferred into a new vial, and centrifuged again to pellet the particle/protein complexes; this procedure was repeated twice. 1D SDS-PAGE experiments were carried out according to ref [7]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) experiments were by a Dionex Ultimate 3000 NanoLC (Dionex, Sunnyvale, CA, USA), equipped with a degasser and a thermostatted microwell-plate autosampler. Further details can be found elsewhere [8].

2.4 CLSM

CLSM experiments were performed using a Leica TCS SP5 inverted confocal microscope (Leica Microsystems AG, Wetzlar, Germany), interfaced with an Ar laser for excitation at 458, 476, 488, and 514 nm, and with a He-Ne laser for excitation at 543 and 633 nm. Glass-bottom petri dishes containing transfected cells were mounted in a thermostated chamber (Leica Microsystems) and viewed with a 40 × 1.25 numerical aperture oil immersion objective (Leica Microsystems). Live cell imaging was always performed at 37 °C. the images were collected using 0.65–10 kw/cm^2 excitation power at the sample and monitoring the emission by means of the Acousto-Optical Beam Splitter (AOBS)-based built-in detectors of the confocal microscopes.

2.5 Flow cytometry

Cells were seeded 24 hours before the experiment in WillCo-dishes to reach a 70% confluence. All treatments were performed in a total volume of 1 ml culture medium. After treatment, cells were washed three times in PBS buffer before trypsination for 1 minute in 0.25% trypsin solution (Invitrogen, Stockholm, Sweden). Cells were centrifuged at 1200 rpm for 5 minutes, and cell pellets dissolved in 500 μl PBS before mesaurement. Internalization of lipoplexes into MDA-MB-435S and MCF7 cells was evaluated with a 635 nm excitation laser (filter 655-730). For all the tested HP concentrations, N=2 independent experiments were conducted, each of which in triplicate. $1.5x10^4$ cells per sample have been acquired in triplicate. MACSQuant® analyzer has been used to run samples and MACSQuantify software to analyze data.

3 RESULTS AND DISCUSSION

Figure 1 shows 1D SDS/PAGE gel of DOTAP/DNA lipoplexes after incubation with increasing HP concentrations from 2.5 to 50%. This allowed us to investigate how the biomolecule corona can change, depending on the biological environment [4, 9]. We observe that the total band intensity of proteins recovered from DOTAP/DNA lipoplexes increases with increasing plasma concentration. Furthermore, the intensity of each protein band seems to follow the same general trend. This evolving trend may indicate that, passing from low to high plasma concentrations, the protein corona of lipoplexes changes in abundance but not in composition. The bands of interest, marked by numbers in Figure 1, were cut from the gel (50%

HP). Then they were processed and analyzed with MS to identify the constituent proteins.

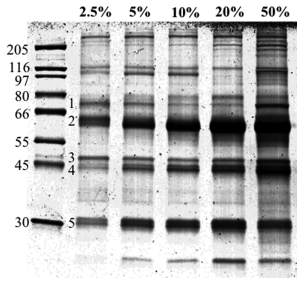

Figure 1. One-dimensional SDS-PAGE gel of HP proteins obtained from DOTAP/DNA lipoplexes. The molecular weights of the proteins on the standard ladder are reported on the left for reference.

We performed a semiquantitative assessment of the protein amounts by the method of spectral counting (SpC). This represents the total number of the MS/MS spectra for all peptides attributed to a matched protein and allows to identify the nature and amount of the most relevant proteins present in the corona. The SpC of each protein identity was normalized to the protein mass and expressed as the relative protein quantity by applying the following equation [4]:

$$NpSpCk = \frac{(SpC / M_w)_k}{\sum_{i=1}^{n} (SpC / M_w)_i} \qquad (1)$$

where NpSpCk is the percentage normalized spectral count for protein k, SpC is the spectral count identified, and Mw is the molecular weight in kDa for protein k. This correction takes into account the protein size and evaluates the real contribution of each protein to the hard corona composition. Normalized SpC (NSpC) values for the most abundant proteins identified in the coronas of DOTAP/DNA lipoplexes are given in Figure 2.

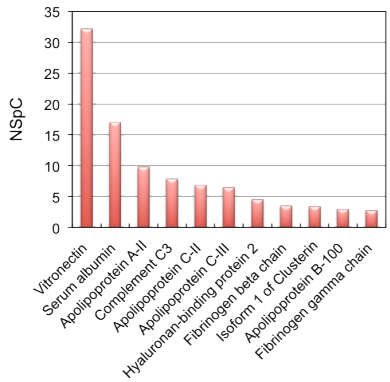

Figure 2. Percentage normalized spectral count (NSpC) values (HP=50%) calculated for each protein hit according to eq 1. Only the most significant hits (NSpC>3) are shown.

It is quite remarkable to notice that vitronectin is largely the most abundant protein in the corona of DOTAP/DNA lipoplexes (≈ 33 % NSpC). Vitronectin is a major ligand for the vitronectin receptor $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins, which are overexpressed in some tumor cell lines [6]. Thus, we asked whether the adsorbed protein corona could dictate a selective access to cells expressing vitronectin receptor, that are $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins.

The distribution of Cy-3 fluorescently tagged DOTAP/DNA/HP complexes was followed in living unfixed MDA-MB-435S by CLSM. At t=4h, punctate cytoplasmic distribution of complexes was observed with minor, if any, accumulation at the plasma membrane (Figure 3A). Characteristic perinuclear localization was also seen (Figure 3B). These findings indicate strong cellular uptake and nuclear translocation of complexes over times typical of transfection experiments.

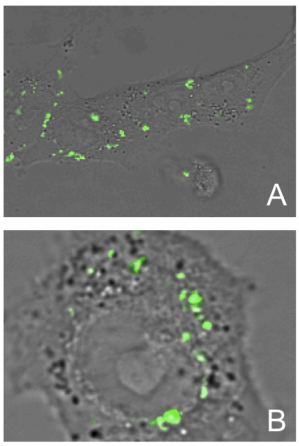

Figure 3. Intracellular distribution of DOTAP/DNA/HP complexes 4h after administration to MDA-MB-435 cells (panel A). In some cases, distinct perinuclear localization of complexes can be seen (panel B).

To quantify cellular uptake of complexes flow cytometry experiments were performed. Results are reported in Figure 4 as relative cellular uptake that is the ratio of fluorescence of DOTAP/DNA/HP complexes to that of bare DOTAP/DNA lipoplexes.

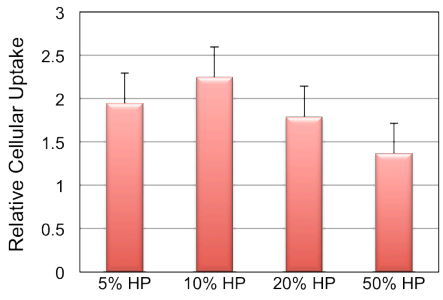

Figure 4. Relative Cellular Uptake of DOTAP/DNA/HP complexes with respect to DOTAP/DNA lipoplexes.

Remarkably, flow cytometry analysis showed that protein corona produced a ≈ 2-fold increase in cellular uptake with respect to untreated lipoplexes

On the other side, DOTAP/DNA/HP complexes were poorly uptaken by MCF7 cells (Figure 5a). They largely accumulated at the plasma membrane (Figure 5b). FACS does not discriminate between membrane-bound and internalized fluorochrome [10]. For that reason, cellular uptake of DOTAP/DNA/HP complexes was not evaluated in MCF7 cells.

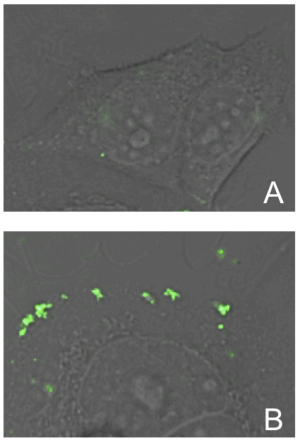

Figure 5. 4h after administration to MCF7 cells DOTAP/DNA/HP complexes were poorly uptaken (panel A). In most cases, they largely accumulated at the plasma membrane (panel B).

4 CONCLUSIONS

Collectively, proteomics, CLSM and flow cytometry experiments suggest that the protein corona plays an important role in the uptake of lipoplexes most probably by proteins (vitronectin) that are specifically recognized by cancer cell receptors (integrins). In the next future, the protein corona might be used as a tool for cancer targeting. Nanoparticles that, once in the blood, become covered of specific proteins recognized by cancer cell receptors could be efficiently uptaken by targeted cells (Figure 6). This "corona targeting" may let us rethink current strategies of active targeting [3-4]. As evident, a deep knowledge of the relationship between surface properties of nanoparticles and composition of the 'protein corona' is the first and fundamental step towards that direction. Obviously, to fully support this intriguing suggestion, more experimental data are needed. For instance, integrin knock-out might help to better individuate their role in the corona-mediated uptake of lipoplexes. Moreover, the existence and relevance of passive mechanims of cellular upatke related, for instance, to particle size and morphology of complexes must be also evaluated. This is currently under investigation in our laboratories.

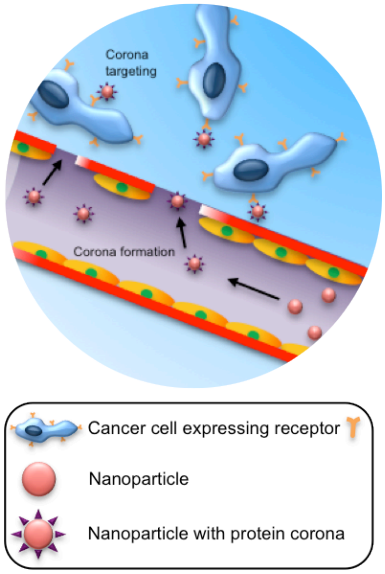

Figure 6. "Corona targeting": proteins in the nanoparticle protein corona recognize receptors that are overexpressed on the surface of cancer cells.

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