Liliana Czernek

INVESTIGATIONS ON THE ROLE OF CANCER-DERIVED EXOSOMES IN INTERCELLULAR COMMUNICATION

"Badanie roli egzosomów pochodzenia nowotworowego w komunikacji międzykomórkowej"

Centrum Badań Molekularnych i Makromolekularnych PAN

ABSTRACT

Cancer is a multifactorial disease involving complex interactions between normal and cancer cells. Recognizing and destroying tumour cells in our body is one of the immune systems actions, but tumours can send signals to the immune system by cell-cell contacts and factors secreted into the extracellular space to escape destruction. In addition, small membrane vesicles released to extracellular space known as exosomes are secreted by normal and cancer cells, thus they are present in most biological fluids. Exosomes can serve as vehicles for the transfer of proteins and RNAs to distant locations and thus act as intercellular messengers involved in cell-to-cell communication. Cancer cells take advantage of this special feature and produce cancer-derived exosomes to support the survival and progression of tumours in many ways and also to contribute to the neutralization of the anti-cancer immune response. Secretion of exosomes involved in intercellular communication by cancer cells to develop the immunosuppression is a line of defence against the body's immune system response. However, the mechanisms underlying these process are poorly understood. Therefore, it is important to learn the routes of cancer immune escape, which will allow to develop more effective anti-cancer treatments and, moreover, adopt methods used by the cancer to evade immune response for treatment of other disorders such as autoimmune diseases or in transplant rejections. The overall aim of this thesis is to investigate the role of cancer-derived exosomes in intercellular communication.

For this purpose, in my doctoral dissertation basic studies of exosomes are included, their characterisation and optimization of production. In these experiments, exosomes were isolated from three cancer cell lines from various cancer types including two melanoma cell lines – A375 and 1205Lu, and a human ovarian cancer cell line developed in our institute (OvC16). It is known that cell culture supernatants contain several types of shed membrane fragments and vesicles; therefore, before performing any functional analysis, it is critical to ensure that the isolated vesicles are exosomes. For this purpose exosomes were characterised by transmembrane markers – CD9 and CD63, and visualised by different approaches like Atomic Force Microscopy (AFM), Transmission Electron Microscopy (TEM) or Nanoparticle Tracking Analysis (NTA). Positive results confirmed that pellets obtained by the differential centrifugation of cell culture supernatants are enriched in exosomes. For the optimisation of exosome production storage conditions, number of purification

steps, but also cell confluency and length of incubation for the exosome production after 1, 2, 3 days of cell culture were examined. Using flow cytometry it was shown that deep freezing alters the quality of exosomes, that purity of exosomes may influence the experimental results and double-purified exosomes should be used. The exosome production depended on cell density (the higher cell density, the more exosomes were produced), but there were no significant differences in exosome production after 1, 2 or 3 days of cell culture conducted at the same (high) confluency. In addition, exosome surface antigens were studied showing that they highly expressed the following antigens: CD47, CD80, CD14, HLA-ABC, but low levels of CD11b antigen.

For the investigation of cell-cell communication via exosomes a method for the visualisation of vesicle uptake was needed. For this purpose vesicles were labelled with fluorescent dyes, either by the well-known carboxyfluoresceine diacetate succinimidyl-ester (CFSE) or by DSSN+ (4,4'-bis(4'-(N,N-bis(6''-(N,N,N-trimethylammonium)hexyl)-styryl)stilbene tetra-iodide), which had not been used for exosome labelling before. In general experiments was determined whether exosomes can be labelled by fluorescent dyes and transfer fluorescence to recipient cells. The transfer of fluorescent dyes contained in exosomes was confirmed by fluorescence microscope and flow cytometer analyses. The intensity of CFSE and DSSN+ fluorescence transferred by labelled exosomes in target cells was determined at various time points. When the DSSN+ dye was used, the maximum fluorescence was already reached after 1h. In contrast, using exosomes labelled by CFSE the highest fluorescence was observed 24h after administration to the cells. These experiments created a foundation for key investigations of cell-cell communication between cancer and immune cells, more precise the study of uptake of cancer-derived exosomes by different immune cells. The question came up whether the differentiation status of the myeloid cells influences exosome uptake. The results showed that macrophages and mature dendritic cells more efficiently took up exosomes than monocytes or immature dendritic cells what indicated that the cell differentiation status affects the efficiency of exosome uptake.

The next step of the work was to determine whether cancer exosomes can modify immune signalling pathways. For this aim the transwell experiments and Western blotting were performed. In a typical transwell study cancer cells were co-incubated with monocytes separated by a membrane with defined pore size. The assumption was that cancer-derived exosomes can alter the expression of immune receptors on monocytes, thus the surface antigens were studied by flow cytometry. Obtained results showed decreased expression of the HLA-DR antigen presenting receptor, involved in immune cell signalling, suggesting an immunosuppressive role for the content – including miRNAs – transported by cancer-derived exosomes. To further study whether cancer-derived exosomes change the expression level of proteins involved in immune suppression, the Western Blot method

was used. miRNAs may impair the target cells by inhibiting the expression of certain target genes resulting in a low level or complete loss of the protein production. To verify that exosomes can induce silencing of immune signalling proteins, different types of cells were incubated with various doses of exosomes. Then the level of NFAT5 protein was determined. NFAT5 is a member of the nuclear factor of activated T cells (NFAT) family of transcription factors, which causes the activation of target genes in the Toll-like receptor (TLR)-induced immune response. TLRs recognize exogenous pathogenic microorganisms and induce innate immune responses that are essential for the host defence, including cytokine production and initiation of an adaptive immune response. The investigated target protein was chosen based on a previous MicroArray analysis of miRNAs found in exosomes. By bioinformatics analysis the NFAT5 mRNA was identified as targeted by the largest number of exosomal miRNAs. Western Blot measurement demonstrated that NFAT5 is expressed in T cells (MOLT4), HeLa and OvC16 cell lines and that exosomes interfere with NFAT5 protein level. In conclusion, by influencing the level of proteins involved in the immune response, cancer-derived exosomes are able to alter immune cell functions and potentially contribute to tumour immune escape.

The milieu and interactions among tumour-associated cells are essential for tumour growth and development. Exosomes seem to take part in cell-cell communication between cancer cells and immune cells and might change cytokine production. IL-12 alone can induce potent antitumor activity or act synergistically with several other immune regulatory cytokines enhancing the antitumor activity of the body. Therefore, the level of IL-12 of immune cells after exosome administration was examined. Results showed lower levels of IL-12 after exosome exposure which may be an additional mechanism by which tumour cells acquire control over the immune system through suppressing the production and secretion of immune stimulatory signals.

The potential of exosomes as therapeutic systems is increasingly recognized. In contrast to cell based therapies exosomes seem to have a lot of advantages e.g. they are easier to store, the loaded content is protected from degradation, and they are more stable in the blood. Exosomes could provide a new source of effective delivery vehicles for drugs or therapeutic nucleic acids, but efficient loading methods still need to be developed. Therefore, examination of exosomes as possible drug carriers was the next part of my work. The research was based on testing different methods of loading anti-cancer compounds into exosomes. The loading efficiency was monitored by MTT and flow cytometry methods. Results indicated that exosomes can function as potential drug carriers for anti-cancer drugs.

It is not fully understood how cancer-derived exosomes are able to induce alterations of immune cell functions and a deeper insight into the cellular and molecular mechanisms underlying tumour immune escape using exosomes may finally lead to novel therapeutic approaches for the benefit of cancer patients. Cancer-derived exosomes contribute to the neutralization of the anti-cancer immune response and assist cancer to acquire control over the immune system. My results confirm that cancer-derived exosomes act as cell–cell communicators and are able to induce alterations of immune cell functions.

The experiments were carried out as part of the ended OPUS 5 project financed by the National Science Centre (NCN 2012/05 / B / NZ2 / 00574) and within the currently running OPUS 10 project financed by the National Science Centre (NCN 2016/21/B/NZ7/02747), whose head is dr hab. Markus Düchler Prof. CBMiM, as well as a project for a young scientist awarded to me in 2015 under the title "Cancer–derived exosomes influence the level of immune signalling proteins".