Evidence for an interaction between the effects of extracellular vesicles and cytokines.

PhD Thesis

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Introduction

Extracellular vesicles (EVs) are small, membrane-surrounded structures secreted by all cells. They have a diameter of few 100 nm and they are of fundamental importance in intercellular communication. More and more evidence is available on the importance of this feature of EVs in the pathogenesis of various diseases as well. Altered EV production by disease-relevant cells has already offered special opportunities for the discovery of new, EV-based diagnostic biomarkers as an alternative for current protein biomarkers. On the other hand, understanding the mechanism by which EVs mediate intercellular communication is also becoming more and more important as several potential future EV-related therapeutic applications EVs are currently being investigated.

Among the numerous biological processes influenced by EVs, blood clotting, development of the neural system and antigen presentation are only a few examples. This multifold effect of EVs can be attributed to the complex structure and diverse constituents of EVs. The large surface area of EVs membranes has its biological importance. The protein (both receptors and ligands) and nucleic acid cargos ensure an even more specific effect.

Regarding the immunological role of EVs, their impact on antigen presentation is among the most intriguing discoveries. During this process, however, EVs do not act by themselves, but in concert with soluble mediators (e.g. hormones and cytokines) found in the same extracellular compartment where EVs are present.

Despite the many shared features of EVs and soluble mediators (including their simultaneous presence in the same extracellular compartment) predisposing for interference, the typical experimental setup used to investigate one factor ignores the possible impact of the other on the results. When studying the biological effect of EVs, up till now little care has been taken of the presence of the soluble mediators. Similarly, the presence or absence of EVs might impact experimental results obtained when assessing the biological effects of cytokines. This raises the question whether results obtained in these studies are physiologically relevant in a multicellular organism where intercellular communication utilizes both pathways.

Furthermore, EV-related studies typically focus on only one type of EVs, although EVs of diverse origin and morphology are present simultaneously in physiological bodily fluids. Our research group was among the first ones recognizing this, and we compared the effects of different types of EV trying to introduce a more global approach to the investigation of EVs than what has been used before. These efforts were later also extended towards non-EV mediators of the extracellular compartment, like cytokines and the possible interaction between EVs and specific cytokines.

Aims of the study

According to our hypothesis, the simultaneous presence of EVs and cytokines results in different biological consequences than their individual presence. This hypothesis was investigated by a global approach, using EVs derived from CCRF T cell line, recombinant TNF as a model cytokine and gene expression changes of U937 monocytic cells as an indicator of biological effect. Using this experimental setup, we attempted to answer the following questions:

- Can EVs be detected in conditioned cell culture media of CCRF T cells? If yes, what types of EVs?
- Can EVs be depleted from conditioned cell culture media of CCRF cells?
- Is there any TNF in the conditioned cell culture media of CCRF cells or EV pellets isolated from this source?
- How does the presence of TNF, T cell derived EVs or both mediators affect global gene expression of U937 cells?
- Are the observed changed reproducible on the level of individual genes or gene products?
- Are the observed changes reproducible in the presence of isolated EV populations?
- What is the biological relevance of the observed changes?
- Is the signal transduction pathway of TNF affected by the protein cargo of EVs?

- Is there a single gene regulatory molecule (transcription factor or miRNA) associated to EVs that might be responsible for gene expression changes observed in the presence of EVs?

Methods

The source of EVs was the conditioned cell culture medium of the CCRF human T cell line, while the U937 human monocytic cell line was used as a recipient cell line. Recombinant human TNF was used as a model cytokine. It was added to EV-rich or EV-depleted conditioned cell culture media in order to study the combined and individual effects.

T cell-derived EVs where isolated according to standard differential centrifugation and ultracentrifugation protocols. The resulting EV samples where characterized using transmission electron microscopy, flow cytometry and Tunable Resistive Pulse Sensing Izon qNano).

Global gene expression changes in the presence of EVs, TNF or both mediators, where assessed by a gene expression microarray and validated by qRT-PCR in case of biologically relevant genes with significant changes. A typical biological function related to these changes, chemotaxis, was also investigated in a Boyden chamber.

Results of the microarray experiment where analyzed using GeneSpring software, Panther GO, GeneMania and GSEA. In silico analysis of EV and MV cargo was based on the data available in the EVpedia database, as well as on our own dataset derived from a meta-analysis of the literature. Protein-protein interactions where obtained

primarily from the Reactome database, while gene regulatory data from TRRUST, RegNetwork and miRWalk 2.0 databases.

Data management, analysis and visualization was reinforced by Python 2.7 scripts, utilizing functions of the BioPython, SciPy and Matplotlib libraries. Connections between genes and proteins where visualized in Cytoscape 3.4.0 with the help of Enrichment Map and Venn and Euler Diagrams plugins. Statistical analysis experimental data was done using GraphPad Prism v4. Molecular modeling of EVs was aided by Packmol, Swiss-Model and PymMol.

Results

Characterization of CCRF T cell line derived-EVs showed the predominance of a 350 nm sized EV population compatible with the features of MVs. Even the individual presence of EVs did influence the expression of numerous genes related to inflammation, as well as to signal transduction. When TNF was simultaneously present, however, an additive interaction could be observed in the case of these genes, as well as an antagonism in case of other immune-related genes. The impact of EVs on the gene expression of SMPD3, CD36 and CNR2 has been observed in multiple independent experiments.

Using a more global approach for the analysis of microarray data (GSEA), gene expression patterns observed in the presence of EVs where found to be related to inflammatory response, the JAK/STAT cascade and the NFκB pathway. In the simultaneous presence of EVs and TNF, the expression of IL-8, CCL2, CXCL10, CD82, ICAM1, NPC1 and GPR68 genes where significantly differentially regulated as

compared to the individual presence of TNF by GSEA. This result has been confirmed by independent experiments in case of IL-8, CCL2, CD82, ICAM1 and NPC1.

The combinatorial effect of the simultaneous presence of EVs and TNF could be reproduced on the level of the IL-8 protein as well. The observed interaction could also be confirmed when using isolated MVs. Furthermore, the combined effect of EVs and TNF was found to be even more evident in case of IL-8 production, when isolated MVs and TNF were resuspended in fresh RPMI cell culture media instead of conditioned cell culture media of CCRF cell line.

In an attempt to identify the vesicular component most relevant to the changes observed in the presence of EVs, we conducted a meta-analysis of the literature for proteins detected as EV cargo. This dataset was compared to the data available in EVpedia and there was a substantial, but not total overlap. In both cases, a significant portion of proteins where related to virus life cycle and to a lesser extent to immunological functions, and signal transduction. The TNF pathway was only slightly affected, however. Protein-protein interactions of EV cargo showed a balance of inhibitory and stimulatory interactions with proteins related to the majority of functional terms in the Reactome database. Among the few functional categories where this balance has been found to be skewed, TNF pathway could be found as well. Here, the balance between signaling from TNFR1 and TNFR2 was affected.

Gene regulatory interactions of EV cargo proteins and miRNA have also been investigated. Gene regulatory EV proteins with the most

pronounced mean changes observed in our experimental setup where TSG101, enolase and members of the Ku autoantigen complex. With a similar approach, miRNA molecules hsa-miR-3676-3p, hsa-miR-1287-3p, hsa-miR-431-3p, hsa-miR-147b and hsa-miR-6829-5p were found to be the most relevant to our experimental results. These miRNAs have not yet been detected from EV cargo, however.

Finally, the results of the GSEA analysis have also suggested that gene expression changes observed in the presence of EVs are closely related to the pattern observed earlier in the presence of oxidized phospholipids, a component that is not typically regarded as cargo of the EVs, but rather a structural component of them.

Conclusions

Our research group has been among the first ones to study the combined effect of diverse vesicular and non-vesicular mediators in the extracellular compartment. According to the results of the present hypothesis-free experimental setup, where EVs where not preselected, we found an interaction between the important soluble mediator TNF and EVs (in particular MVs). Multiple independent experiments with EV samples isolated by different techniques have provided evidence for this interaction on the gene expression level, as well as on the protein level in the case of the paramount chemokine IL-8.

Based on gene expression data observed in the presence of EVs, this interaction is complex. From a functional perspective, the presence of EVs does affect signal transduction pathways, as well as

inflammatory process. As a result, EVs might have an important role both in facilitating the initiation and limiting the extent of inflammation.

The observed changes might be attributed to the complex nature of EV cargo. Although no single component of EVs could be identified to be solely responsible for the observed effects by our bioinformatical analysis of EV cargo, a handful of proteins and miRNAs have been identified that might be further investigated for being responsible for some of the EV effects.

Given the evidence for an interaction between EVs and cytokines, we can conclude that the role of EVs should not be ignored when evaluating the effects (and clinical importance) of cytokines. This also raises the question whether our current understanding of the effect of soluble mediators should be reevaluated in light of the possible soluble mediators- EV interactions. This reevaluation might lead to a better understanding of the mechanism of action of numerous biological therapies targeting cytokines as well, and may thus, indirectly have an impact on clinical practice or help to discover better therapeutic options.

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