Analysis of extracellular vesicle-associated DNA release induced by antibiotic exposure

Doctoral Theses

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Introduction

Extracellular vesicles (EVs) play key roles in intercellular communication by which they may impact a wide range of biological functions of cells. The biological roles of EVs have attracted substantial attention in various fields of biomedicine. EVs are phospholipid bilayer enclosed particles that can deliver lipids, proteins, nucleic acids (RNA, DNA) and metabolites to both neighboring and distant cells. EVs are heterogeneous in their biogenesis, molecular composition and size. Exosomes (EXOs) are released from cells during the exocytosis of multivesicular bodies. EXOs typically represent the smallest sized (~100 nm) EVs. Microvesicles (MVs) are usually intermediate-sized vesicles (~100–1000 nm) and they shed from the cell surface upon outward budding of the plasma membrane. Large vesicles with diameter >1 µm can be produced during apoptosis (in which case they are referred to as apoptotic bodies, APOs). Of note, highly migratory tumor cells also release large vesicles (referred to as large oncosomes) of several µm in diameter. EVs are released from resting cells, however, different cellular and environmental stimuli (e.g. activation or apoptosis induction) can increase the amount of secreted EVs and can modify their content. Vesicular proteins and nucleic acids are considered as potential new biomarkers that can lead to early diagnostics of pathological conditions. However, in order to develop EV-based molecular diagnostic procedures, it is essential to understand the content and functional effects of different cell-derived EVs.

EVs can alter signaling of recipient cells by either cell surface receptor-ligand interactions or upon uptake by cells. EVs have been shown to deliver specific RNA and DNA sequences to healthy cells. Thus, they can modify the genetic composition of recipient cells and alter their functions. EXOs have been shown to carry DNase-resistant intravesicular DNA, protected by the phospholipid bilayer membrane. Until now, most studies focused exclusively on intra-exosomal DNA, and DNase digestion was mainly used to eliminate any potential contaminating extravesicular DNA. In our study we focused on the analysis of EV-associated intra- and extravesicular DNA.

We investigated the impact of sustained exposure of cells to the fluoroquinolone antibiotic ciprofloxacin on the released EVs, with emphasis on the DNA content. Ciprofloxacin is widely used in humans against bacterial infections as well as in cell cultures against *Mycoplasma* contamination. Ciprofloxacin inhibits both the bacterial DNA gyrase and the mammalian topoisomerase II enzymes responsible for proper DNA replication. Given that

ciprofloxacin mainly inhibits the mitochondrial isoform of mammalian topoisomerase II, its presence induces mitochondrial DNA (mtDNA) fragmentation as well as subsequent gradual decrease in mtDNA content. Consequently, ciprofloxacin treatment induces oxidative stress and mitochondrial dysfunction in mammalian cells. Importantly, the presence of ciprofloxacin has been also reported to lead to aneuploidy caused by the genotoxic stress of mammalian cells.

We hypothesized that these effects induced by ciprofloxacin could alter the DNA content of EVs, the analysis of this question was the main subject of this Ph.D. dissertation. We also wondered if activation or apoptosis induction had an impact on the association of DNA with EVs of ciprofloxacin-exposed Jurkat cells.

Objectives

In our current work we asked the question whether a sustained (>14 days) exposure to a quinolone antibiotic, ciprofloxacin had an impact on the released EVs of Jurkat cells.

1. ***Analysis of ciprofloxacin effects on Jurkat, MiaPaCa and U937 cells and on the released EVs:***
    - Analysis of viability of ciprofloxacin-exposed cells by flow cytometry
    - Proteomic analysis of ciprofloxacin-exposed Jurkat cells
    - Isolation of cell-derived EVs and their characterization by flow cytometry

2. ***Detection of EV-associated DNA secreted from ciprofloxacin-exposed Jurkat cells***
    - DNA content analysis of EVs before and after DNase I digestion
    - Analysis of extravesicular DNA associated with EXOs by Optiprep density gradient ultracentrifugation and by elution with high salt concentration buffer

3. ***Analysis of ciprofloxacin-exposed Jurkat cell-derived EVs by an optical biosensor:***
    - Label-free analysis of EV adhesion onto a fibronectin-coated surface
    - Adhesion of EXOs before and after DNase I digestion onto a fibronectin-coated surface

4. ***Analysis of ciprofloxacin-exposed control, activated and apoptotic Jurkat cell-derived EVs:***
    - Optimizing conditions for cellular activation and apoptosis induction, proteomic analysis of cells by mass spectrometry
    - Size distribution and concentration measurements of EVs by tunable resistive pulse sensing and by transmission electron microscopy
    - Isolation of EVs before and after DNase I digestion, nuclear and mitochondrial DNA analysis of EVs
    - Proteomic analysis of EVs by mass spectrometry

5. ***Data analysis for the EV-TRACK knowledgebase, summarizing experimental results, conclusions to further improvement of the knowledgebase***

Methods

Cell cultures

We cultured Jurkat human T-cell lymphoma, U937 human histiocytic lymphoma cell lines and MiaPaCa pancreatic cancer cells in order to obtain EVs. Jurkat and U937 cells were cultured in RPMI medium, whereas MiaPaCa cells were grown in DMEM medium, both containing 10% fetal bovine serum, 2 mM glutamine, 0.5% AB/AM (Antibiotic-Antimycotic 100X), with or without 10 µg/mL ciprofloxacin (>14 days). Cells were cultured for 6 or 24 hours under serum-free condition depending on the experiments. For EV isolation, Jurkat and U937 cells were cultured at a density of 8 x 10^5 cells/mL, while MiaPaCa cells were grown at a confluence of 90%.

Isolation of extracellular vesicles

A combination of multistep differential centrifugation and hydrostatic filtration was used for EV isolation, detailed in Table 1. The APO pellet was re-suspended in PBS, and filtered by gravity through a 5 µm filter. The EV pellets were washed once in PBS to obtain pure vesicle samples. In order to obtain EXOs of higher purity, Optiprep™ density gradient centrifugation was applied. Discontinuous Optiprep™ gradient was prepared by layering 40%, 20%, 10% and 5% iodixanol on top of each other. Isolated EXOs were overlaid onto the top of the gradient and were pelleted at 100,000 g for 18 hours with a MLS-50 rotor (Beckman Coulter). After ultracentrifugation, 9 individual 0.5 mL fractions were collected manually from the top of the gradient and washed further in PBS (100,000g, 3 hours, 4°C).

Table 1: Summary of the applied EV isolation methods and their parameters.

Step	Method	Parameters	EV fraction
1.	centrifugation	300g, 10 min, 25°C	cell pellet
2.	centrifugation	2000g, 20 min, 16°C	APO pellet
3.	hydrostatic filtration	0,8 µm	-
4.	centrifugation	12500g, 40 min, 16°C	MV pellet
5.	hydrostatic filtration	0,2 µm	-
6.	ultracentrifugation	100000g, 70 min, 4°C	EXO pellet

Activation and apoptosis induction of Jurkat cells

Conditions for cellular activation and apoptosis induction were selected by pilot experiments. For EV isolation and characterization, we selected 0.5 µM staurosporine, and 0.1 µM A23187 ionophore in combination with 20 ng/mL PMA (phorbol-12-myristrate-13-acetate) for apoptosis induction and cell activation, respectively. EVs were isolated from the apoptotic or activated cell supernatant after 6 hours of incubation.

Fluorescent microscopy of cells

Upon ciprofloxacin exposure, steady-state, activated and apoptotic states of Jurkat cells were documented using a digital fluorescent microscope (EVOS FL Color Imaging System, Thermo Fisher Scientific). Cells were stained with the membrane dye PKH67 and with the DNA dye DAPI in order to analyze cellular morphology.

Flow cytometry of cells and extracellular vesicles

Viability of Jurkat cells was tested regularly to obtain the ratio of viable, apoptotic and necrotic cells. Cells were also analyzed in details to characterize apoptotic and activated cell states. Cells were stained with annexinV-FITC and propidium iodide (PI) and were measured by flow cytometry (using a FACS Calibur flow cytometer), by which the following cell populations could be separated from each other: viable, early apoptotic, late apoptotic and necrotic cells.

Both Jurkat cells and cell-derived EVs (APOs, MVs and EXOs) were stained with annexinV-FITC and PI. Moreover, EVs were also stained with anti-CD63-PE and histone specific anti-H2B-FITC antibodies. The staining and the measurement of MVs and APOs were carried out directly, whereas EXOs were conjugated onto the surface of latex beads (lat-EXO) before analysis. To verify the vesicular nature of EVs and to exclude the presence of protein aggregates, we carried out differential detergent lysis of EVs by addition of Triton X-100 in a 0.1% final concentration to the samples.

Analysis of vesicular DNA

DNase I digested and non-digested EV samples were analyzed by flow cytometry or were subjected to DNA analysis. The DNA content of EVs was purified using a Genomic DNA Mini Kit (Geneaid) according to the instructions of the manufacturer. DNA concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The presence of nuclear (*GAPDH, p53*) and mitochondrial (control region, *RNR1*) DNA in APO,

MV and EXO samples were evaluated after polymerase chain reaction using agarose gels. Purified DNA content was also analyzed directly using agarose gels or microfluidic chips (12,000 DNA Chip, Bioanalyzer 2100; Agilent Technologies).

Mass spectrometry of cells and extracellular vesicles

The protein composition of Jurkat cells cultured in the presence or absence of ciprofloxacin were studied by mass spectrometry. Furthermore, ciprofloxacin-exposed Jurkat cells of different functional conditions (activation and apoptosis) and the respective secreted EVs were subjected to proteomic analysis. The identification of peptides and proteins were validated and visualized by the Scaffold 4.5.1. Software.

Label-free optical analysis of surface adhesion of extracellular vesicles

The adhesion of ciprofloxacin-exposed Jurkat cell-derived EVs onto a fibronectin-coated surface was characterized by the Epic® Benchtop System (Corning), a novel label-free optical biosensor. First, we optimized the experimental conditions for proper biosensor immobilization with the fibronectin. Next, our aim was to compare the binding properties of the different EV subsets (APOs, MVs and EXOs) onto fibronectin surfaces. We also analyzed whether DNase I treatment affected EXO adhesion onto fibronectin.

Size distribution and concentration of the different extracellular vesicle subsets

Size-based EV fractions released by ciprofloxacin-exposed Jurkat cells of different functional states were submitted to tunable resistive pulse sensing analysis using a qNano instrument (IZON Science). By applying qNano, size distribution and concentration of the EV subsets were determined. Calibration was performed by using known concentrations of different sized beads.

Transmission electron microscopy of extracellular vesicles

In order to characterize the morphology and size of the different EV fractions, EV pellets were fixed with 4% paraformaldehyde and 2% glutaraldehyde in PBS for at least 60 min at room temperature and analyzed by transmission electron microscopy.

Results

Effect of ciprofloxacin exposure of Jurkat cells

In the present study we investigated the effect of prolonged exposure of Jurkat cells to a fluoroquinolone antibiotic, ciprofloxacin. We first compared Jurkat cells with or without a sustained (>14 days) exposure to ciprofloxacin. In line with previous observations by others, we found that the presence of low-dose (10 µg/mL) of this antibiotic did not have a significant effect on cell viability. Moreover, also in agreement with previous published findings, our mass spectrometry analysis of cells showed that the presence of ciprofloxacin resulted in a slightly elevated percentage of cellular proteins associated with oxidative stress and defense responses, mitochondrial degradation, and in a somewhat reduced percentage of respiratory electron transport chain-associated proteins. Of note, all the observed minute proteomic differences were in line with previously published data, and were found reproducibly in two independent experiments.

Next, we showed that ciprofloxacin-exposed Jurkat cells (but not those cultured without this antibiotic) released EXOs with substantial PI and anti-histone H2B staining, suggesting an EXO-associated DNA and histone H2B secretion. In order to study if the observed effect of ciprofloxacin was specific to Jurkat cells, EVs were isolated from MiaPaCa and U937 cells, and were analyzed by flow cytometry. Our results showed that the presence of ciprofloxacin induced a robust EXO-associated DNA secretion by MiaPaCa cells. However, we did not observe this phenomenon in the case of U937 cells, suggesting a cell-specific mechanism of ciprofloxacin.

Next, we compared the amount of ciprofloxacin-induced DNA of the Jurkat cell-derived EV samples. We found that all EV fractions carried DNA. However, DNA was mainly associated with the EV fraction containing EXOs. Furthermore, our results show that the amount of EXO fraction-associated DNA decreased significantly upon digestion of the EXOs with DNase I, which proves the presence of DNA outside of the vesicles (extravesicular DNA). To confirm that the DNA in the EXO pellet was indeed associated with the surface of EXOs (rather than being inside of them), we ran our samples on an Optiprep^{TM} density gradient. We found co-localization of DNA and EXOs within the same density gradient fraction, suggesting the binding of DNA to the exosomal membrane. Next, we tested if the EXO-associated DNA could be eluted from the surface of EXOs in the presence of high salt concentration (2 M NaCl). Our results showed a weakened DNA

association with the surface of EXOs after applying high salt concentration washing step of EXOs. Based on these experiments we can conclude that DNA binding to the external surface of EXOs involved by electrostatic interactions.

Thereafter, we tested the adherence of ciprofloxacin exposed Jurkat cell-derived EVs using a label-free optical biosensor. Our results show that APOs and MVs did not bind to the fibronectin coating of the biosensors. In sharp contrast, EXOs adhered significantly onto the fibronectin-coated surfaces. Next, we tested the possibility that exofacial DNA on EXOs could play a role in this EXO-fibronectin interaction. The results show that adhesion of EXOs onto fibronectin was reduced significantly upon DNase I digestion, which suggests a functional significance of the EV-associated DNA.

Effect of activation and apoptosis induction on ciprofloxacin-exposed Jurkat cells

We also wondered if activation or apoptosis induction had an impact on the association of DNA with EVs of ciprofloxacin-exposed Jurkat cells. Cell activation and apoptosis induction were accompanied by an alteration in the amount of secreted EVs and in their protein composition. We found that activation of Jurkat cells increased the amount of released EXOs and their protein content, whereas apoptosis induction rather resulted in an increased MV and APO secretion.

We detected mtDNA in all EV fractions (APO, MV and EXO) by polymerase chain reaction and agarose gel electrophoresis. In contrast, genomial DNA sequences were only found in EXO samples. The mitochondrial and genomial DNA sequences were sensitive to DNase I digestion of EXOs, proving the association of DNA with the external surface of EXOs. DNase I sensitive DNA sequences were also detected in EVs derived from Jurkat cells upon cellular activation or apoptosis induction.

Our flow cytometric analysis showed that annexinV-FITC staining of EVs diminished after lysis with 0.1% Triton X-100, indicating the presence of detergent-sensitive membrane-enclosed vesicular structures. Moreover, detergent lysis-resistant DNA staining of APO and EXO samples was observed, which may suggest that the association of DNA with EVs was stabilized by protein-protein interactions.

Ciprofloxacin-induced mitochondrial dysfunction of cells was indicated by the increased mtDNA content detected in EV preparations and the presence of mitochondrial proteins identified by mass spectrometry. In addition to the increased amount of DNA in EXO samples, genomic DNA-binding histone proteins were also enriched in the EXO

preparations according to our quantitative mass spectrometry analysis. Moreover, flap endonuclease 1 protein involved in mtDNA binding was identified in EXO samples. The observed DNA-binding protein content of EXOs may also suggests that the association of DNA with EXOs is stabilized by DNA-bound protein complexes. EV-specific proteins (for example CD81 and heat shock proteins in EXOs, integrin and VAMP proteins) were also detected in MVs samples.

EV-TRACK knowledgebase

In addition to our experimental work, I also participated in a data analysis project, which was accomplished by an international consortium called EV-TRACK (Extracellular Vesicle-Transparent Reporting And Centralizing Knowledgebase). Based on the results of data analysis, the EV-TRACK knowledgebase was created. EV-TRACK summarizes various aspects that should be taken into account during EV isolation and characterization and during the publication of the results. Our experimental results described in my PhD thesis, suggest that the EV-TRACK knowledgebase should be complemented with the aspect of DNA analysis of EVs.

Conclusions

Based on our results we conclude that a 10 μg/mL low-dose ciprofloxacin exposure did not initiate cell death, but slightly increased the number of cellular stress-related proteins based on mass spectrometry analysis. As a major finding of our study, we found evidence that the presence of ciprofloxacin had an effect on the molecular composition of EVs secreted by mammalian cells. Ciprofloxacin exposure induced the release of substantial amounts of DNA associated with Jurkat cell-derived EVs (particularly with EXOs). Our experiments also demonstrated the abundance of mtDNA associated with the different types of Jurkat cell-derived EVs. Unexpectedly, we found that Jurkat EXO-associated mtDNA and also genomial DNA were predominantly associated with the external surface of EXOs.

Mitochondria are of endosymbiotic origin, and share numerous features with prokaryotes which may explain the observed mitochondrial susceptibility to the observed antibiotic-induced damage. Intracellular mitochondrial EV formation and fusion of mitochondrial EVs with multivesicular bodies may provide a possible mechanism by which mtDNA gets associated with the surface of EXOs. Of note, a strikingly similar mechanism of bacterial DNA release with bacterial outer membrane vesicles (OMVs) has been documented by Fulsundar *et al.* in 2014. In this publication, antibiotic-induced stress response of bacterial cells was reported to increase the amount of OMV-associated DNA. This OMV-associated DNA also represents a mechanism of horizontal gene transfer of bacteria. However, in mammals, stress-induced release of EXO-associated DNA (such as mtDNA) may rather have an immunomodulatory function.

Our analysis confirmed that DNase I sensitive exofacial DNA played a significant role in binding of EXOs to fibronectin. Binding of DNA-covered EXOs to extracellular matrix proteins (such as fibronectin) may enable antibiotic-induced stressed cells to leave a trail for innate immune cells (similarly to extracellular matrix-bound chemokines).

In summary, our data may suggest a novel EV-related pathway of the removal of damaged mitochondria and genomial DNA from cells. We assume that genomial DNA could be associated with the surface of EXOs due to DNA instability, aneuploidy and DNA breaks induced by ciprofloxacin exposure of cells. Given the broad use of antibiotics worldwide, these data warn for possible previously unregarded effects of sustained antibiotic consumption.

My publications

Publications related to the Doctoral Thesis:

1. **Nemeth, A.**, Orgovan, N., Sodar, B. W., Osteikoetxea, X., Paloczi, K., Szabo-Taylor, K. E., Vukman, K. V., Kittel, A., Turiak, L., Wiener, Z., Toth, S., Drahos, L., Vekey, K., Horvath, R., Buzas, E. I. (2017) Antibiotic-induced release of small extracellular vesicles (exosomes) with surface-associated DNA. Sci Rep, 7: 8202.

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1. Szabo-Taylor, K. E., Toth, E. A., Balogh, A. M., Sodar, B. W., Kadar, L., Paloczi, K., Fekete, N., **Nemeth, A.**, Osteikoetxea, X., Vukman, K. V., Holub, M., Pallinger, E., Nagy, G., Winyard, P. G., Buzas, E. I. (2017) Monocyte activation drives preservation of membrane thiols by promoting release of oxidised membrane moieties via extracellular vesicles. Free Radic Biol Med, 108: 56-65.

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