Focused miRNA expression analysis of heart, brain and cancer cells

Thesis of the Ph.D dissertation

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1. INTRODUCTION

MicroRNAs (miRNAs) are single-stranded short non-coding RNA molecules of about 20-22 nucleotides in length, which usually repress gene expression by binding at the 3'UTR region of target gene. However the function of most miRNAs is not well known, many miRNAs have been found to associate with apoptosis and cancer, suggesting they function as oncogene or tumor suppressor gene. The global role of miRNA function in the heart has also been analyzed, and it has been revealed that miRNAs play an essential role during the heart development. In addition miRNA expression profiling studies demonstrate that expression levels of specific miRNAs change in diseased human hearts, pointing to their involvement in cardiomyopathies. Recent miRNA research advances showed great potential for the development of novel biomarkers and therapeutic targets.

The nutritionally essential polyunsaturated fatty acids (PUFAs) are fatty acids that contain more than one double bond in their backbone. PUFAs are involved in many biological functions including inflammatory response, cell membrane structure and function, cell signaling transduction pathways, regulation of transcription, fetal growth and development, retinal function, and brain development. Small amounts of very long chain PUFA with a chain length of 22 carbons or more are present in many animal tissues, especially in the testis, retina, brain, and liver. There are three major types of PUFAs that are ingested in foods and used by the body: alpha-lipoic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Evidence from cellular and molecular research studies indicates that the cardioprotective and antitumor effects of PUFAs result from a synergism between multiple, intricate mechanisms that involve antiinflammation, proresolving lipid mediators, modulation of cardiac ion channels, reduction of triglycerides, influence on membrane microdomains and downstream cell signaling pathways. However PUFAs are being used for years as a complementary therapy in case of cancer, the molecular mechanisms by which PUFAs protect or enhance tumor development, has still not been fully investigated. It has become necessary to determine the roles of fatty acids in the development of or protection against human cancer.

2. AIMS OF THE STUDY

Our aim was to identify miRNA molecules playing a key role in the mechanism of apoptosis and treatment response in cancer cells and in the action of some special neuron types.

Our experiments can be divided to four groups:

1) Purification of high-quality miRNA from the heart tissue.

Traditionally, miRNA purification relies on organic extraction followed by alcohol precipitation, however, much of the small RNAs are lost during this procedure. Several companies developed special purification kits for miRNAs that employ organic extraction followed by binding and purification of small RNAs on a silica fiber matrix. However reproducible expression analysis of miRNAs is highly dependent on the quality of the RNA, none of the commercially available kits are specialized to fibrotic tissues, such as the myocardial tissue. Therefore our aim was to develop an optimized miRNA purification protocol for fibrosuos tissues.

2) MiRNA and mRNA profile of PUFA treated glioma cells

Previous studies showed that certain polyunsaturated fatty acids, especially γ-linolenic acid (GLA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have tumoricidal action against glioma cells both in vitro and in vivo. Understanding how the signaling pathways involved in surviving and inducing cell death of different glioma cells are regulated during PUFA treatment is important for the development of more effective tumor therapies. To date, no data is available as to the effect of different PUFAs on miRNA expression in glioblastoma cells. Our aim was to analyze the miRNA expression changes of three different glioma cells treated with three different PUFAs (GLA, AA and DHA) and temozolomide as a chemotherapeutic agent currently used in glioma treatment.

3) Effects of dietary polyunsaturated fatty acids on liver and heart miRNA expression.

However several clinical studies have confirmed that PUFAs are efficacious in case of cardivascular, inflammatory and cancerous diseases, it's still unknown how are these influence the gene expression pattern of the cells. Our aim was to identify the gene expression changes and biochemical pathways, that are influenced by PUFA treatment.

4) Classifying electrophysiologically-defined interneurons by single cell gene expression profiling

Individual cells exhibit a large degree of variability in their gene expression profile. Whole-cell patch-clamp recording enables detecting electrophysiological signals from neurons and total RNA can be harvested into the patch pipette from the same cells.

Subsequent nucleic acid amplification techniques can provide gene expression measurements on electrophysiologically characterized cells. Our aim was to optimize methods for determining mRNA or miRNA expression in single neurons after patch-clamp recording at a single molecule level by using high-density nanocapillary digital PCR. With the combination of dPCR and patch-clamp techniques, our aim was to assess mRNA and miRNA expression pattern of three neuron types.

3. MATERIALS AND METHODS

Cell culture and treatment

Glioblastoma cancer cell lines (GBM2, GBM5, U373) were cultured in RPMI-1640 medium and DMEM nutrient mixture medium, respectively, supplemented with 10% fetal calf serum and 100 U/ml penicillin-streptomycin in a 5% CO_2 humidified incubator at 37 °C. The cells were treated with 50 and 100 μM AA, DHA, TMZ, 75 and 150 μM GLA for 24 hours and then harvested with trypsin and washed with PBS.

Holographic Imaging

The survival of the tumour cells was assessed using HoloMonitor™ M3 holographic imaging.The HoloMonitor TM M3 is a cell counter and analyzer which integrates flow cytometry and phase contrast microscopy to advance discovery. Besides counting the cells it can analyze them regarding cell morphology, size, shape and viability.

Improved miRNA isolation protocol for fibrotic tissues

We optimized a miRNA purification protocol based on an existing kit which was originally developed for paraffin embedded tissue preparations, but was not appropriate for fibrotic tissues. Our protocol is an improved version of the High Pure miRNA Isolation Kit (Roche) with inserting several additional steps into the standard protocol. The essence of our new method relies on efficient proteolytic digestion of fibrotic tissue, and a two-step purification protocol, which at first separate the total RNA including miRNAs, then on a new column miRNA is further purified by an additional elution.

Profiling of miRNAs by high-throughput, nanocapillary QRT-PCR

Amplification of the samples was followed in real time with an OpenArray NT Cycler. The OpenArray® System enables thousands real-time qPCR reactions to run on a single

OpenArray plate. Each TaqMan Open Array plate contains 3075 through-holes arranged in 48 subarrays of 64 through-holes each. Each OpenArray slide contained 112 different miRNA Assays which means that 24 samples can be analysed at the same time. cDNA samples (or water for no template controls) were added to a 384-well plate containing universal Taqman master mix for OpenArray amplification. The OpenArray autoloader transfers the cDNA/master mix from the plate to the array through-holes by capillary action.

QRT-PCR

For the confirmation of HTS QRT-PCR miRNA expression data and for the determination of mRNA levels we used traditional QRT-PCR technique. The reverse transcription of the miRNA samples was performed with TaqMan® MicroRNA Reverse Transcription Kit. Each sample was reverse transcribed in the presence of 5X RT TaqMan® MicroRNA Assays. QRT-PCR was performed on the Excicycler instrument (Bioneer) with the TaqMan protocol.

Gene expression measurements with digital PCR

Molecular characterization of the single neurons was performed with digital PCR technique. Digital PCR (dPCR) is a refinement of conventional polymerase chain reaction methods that can be used to directly quantify and clonally amplify nucleic acids.. The key difference between dPCR and traditional PCR lies in the method of measuring nucleic acids amounts, with the former being a more precise method than PCR. PCR carries out one reaction per single sample. dPCR also carries out a single reaction within a sample, however the sample is separated into a large number of partitions and the reaction is carried out in each partition individually. The partitioning of the sample allows one to count the molecules by estimating according to Poisson. As a result, each part will contain "0" or "1" molecules, or a negative or positive reaction, respectively. After PCR amplification, nucleic acids may be quantified by counting the regions that contain PCR end-product, positive reactions. dPCR, however, is not dependent on the number of amplification cycles to determine the initial sample amount, eliminating the reliance on uncertain exponential data to quantify target nucleic acids and providing absolute quantification. For dPCR analysis half (2.5 µl, in case of *rps18*) or the entire (in case of all the other genes) of the RT reaction mixture, 2 µl Taqman® Assays, 10 µl OpenArray® Digital PCR Master Mix (Life Technologies) and nuclease free water (2-4.5 µl) were mixed in a total volume of 20 µl. The mixture was evenly distributed on an OpenArray® plate, and RT mixes were loaded into four wells of a 384-well plate from

which the OpenArray autoloader transfered the cDNA/master mix by capillary action into 256 nanocapillary holes (4 subarrays) on an OpenArray® plate.

Electrophysiology– whole cell patch clamp technique

Young Wistar rats were decapitated, the brains were quickly removed, and 300-µm-thick parasagittal sections of cerebral cortex were prepared. Slices were transferred to a chamber and perfused with ACSF. Whole-cell patch clamp recordings were made from neocortical neurons. At the end of the recording, as much as possible of the cell's content was aspirated into the recording pipette by application of a gentle negative pressure while maintaining the tight seal. The pipette was then delicately removed to allow outside-out patch formation. Next, the content of the pipette was expelled into a test tube and stored or immediately used for RT.

4. RESULTS AND DISCUSSION

4.1 Purification of high-quality micro RNA from the heart tissue

We optimized a miRNA purification protocol based on an existing kit which was originally developed for paraffin embedded tissue preparations, but was not appropriate for fibrotic tissues. Using the same tissue as starting material we made total RNA purification with the traditional Trizol protocol, as well as Trizol protocol but inserting an extra step to proteolitically digest the muscle fibers with Proteinase K treatment. We also used silica-column based, commercially available miRNA purification kit from Roche as a control. The essence of our new method relies on efficient proteolytic digestion of fibrotic tissue and a two-step purification protocol, which at first separate the total RNA including miRNAs, then on a new column miRNA is further purified by an additional elution.MiRNA obtained by our novel protocol gave more reproducible results in quantitative real-time PCR (QRT-PCR) analysis and more significant calls in DNA microarray analysis. that resulted in a significantly higher miRNA yield as compared to other methods in the literature.

4.2 MiRNA profile of PUFA treated glioma cells

We investigated the effects of GLA, DHA and AA that are PUFAs. In order to differentiate specific responses to different PUFAs we analyzed the effect of temozolomide, which is an oral alkylating agent that is used for the treatment of brain tumors. We have

analyzed the expression of 112 microRNA by using high-throughput, nanocapillary QRT-PCR. Most of the miRNA expression changes can be dedicated to the general apoptotic cell death (with all PUFAs as well as temozolomide), such as mir-34, mir-25, mir-17, mir-26a, mir-29c, mir-31, mir-200a, mir-206 in the three cell lines tested. The same general effect, up-regulation of mir-140, mir-323 and mir-133b could be seen but only in U373 cells, but not in GBM2 nor in GBM5 cells. Besides non-specific modification of miRNA expression, we could detect temozolomide and PUFA treatment-specific alterations in miRNA level. In case of temozolomide we found specific up-regulation of mir-182, and down-regulation of mir-16 and mir-183. Mir-143 was found to be repressed and mir-20b was induced by PUFA treatments. The expression of mir-125b was repressed in GBM5 cells only, while, mir-197 was up-regulated in U373 cells. The same cell-type specificity could be observed in the case of mir-206 that was down-regulated in GBM2 cells, but was up-regulated in U373 cells. These differences in the expression of certain types of miRNAs in response to various PUFAs by different glioma cells could be due to differences in the signalling pathways in different types of tumor cell lines, even though the three studied cell lines were glioblastoma cells.

To confirm the importance of miRNA expression regulation during apoptosis, we listed the target genes of most of the miRNAs that were changed upon PUFA or temozolomide treatment. Most of the targets of the differentially expressed miRNAs in response to PUFA treatment were apoptotic genes. It is likely that the exhibited changes in miRNAs are as a consequence of apoptosis. This is supported by the fact that most of the mRNAs that are altered in response to PUFAs and temozolomide as noted in the present study are regulated by 9 miRNAs that can be classified as apoptosis-specific genes. Furthermore, most of the genes are overlapping, suggesting the existence of parallel regulatory pathways. These genes are the following: *irs1*, *irs2*, *cox2*, *ccnd1*, *sirt1*, *tp53inp1*, *itgb3*, *kras* és a *bcl2.*

Understanding how the signaling pathways involved in surviving and inducing cell death of different glioma cells are regulated during PUFA treatment is important for the development of more effective tumor therapies including PUFAs alone or in combination with other drugs. In the present study, we focued on microRNA expression changes, as microRNAs are currently being recognized to have central role in the regulation of the expression of key gene families that are involved in cell physiology and the fate of cancer cells. In conclusion, we could demonstrate that in response to different PUFAs the expression of miRNA and the expression of their target mRNA coding genes were differentially altered. Most of the regulated genes could be classified as apoptotic genes and were up-regulated by PUFAs and temozolomide, while the same treatment resulted in repression of corresponding

miRNAs. From these results, we conclude that PUFAs trigger apoptosis in glioma cells by regulating miRNA and their corresponding gene expressions.

4.3 Effects of dietary polyunsaturated fatty acids on liver and heart miRNA expression

The PUFA experiments were carried out on rats to determine the miRNA expression changes in the heart and liver. We observed significant changes in the expression level of 8 miRNA species. In the heart we observed significant miRNA expression changes in the case of 5 miRNAs (*miR-126, miR-133b, miR-148a, miR-150, miR-200a)*, while in the liver we observed significant changes in the case of the 8 following miRNAs: *miR-21, miR-107, miR-126, miR-133b, miR-148a, miR-200a* and *miR-205.* To confirm our results we determined the expression changes of the target genes of the differentially expressed miRNAs. These targets are vital genes, playing a key role in apoptosis, angiogenesis and lipid metabolism. These genes are the following: *apoa5, irs1, tnf, fas, vegfa, itgb3, sele, selp, ppara, cxcl12, abca1, acox1, pafah1b1, lypla3.*

According to our results *apoa5* and *sele* exhibited a significant repression due to PUFA treatment in the heart. The *apoa5* is an important determinant of plasma triglyceride levels, a major risk factor for coronary artery disease. It is a component of several lipoprotein fractions including VLDL, HDL, chylomicrons.

Selectin-E (*sele*) is expressed only on the surface of endothelial cells. During inflammation, *sele* plays an important part in recruiting leukocytes to the site of injury. In cases of elevated blood glucose levels, such as in sepsis, E-selectin expression is higher than normal, resulting in greater microvascular permeability. E-selectin is also an emerging biomarker for the metastatic potential of some cancers and for hypertonia or atherosclerosis.

In the liver we have shown that PUFA treatment inhibits the expression of selectin P (*selp*). P-selectin plays an essential role in the initial recruitment of leukocytes to the site of injury during inflammation. According to previous studies, induction of *selp* gene predicts inflammation, liver cirrhosis or cancer.

In conclusion, we could demonstrate that in response to different PUFAs the expression of miRNA and the expression of their target mRNA coding genes were differentially altered. Most of the regulated genes are involved in cell adhesion, angiogenesis and lipid metabolism and were up-regulated by PUFAs, while the same treatment resulted in repression of corresponding miRNAs.

4.4 Classifying interneurons by single cell gene expression profiling

Population-average expression survey of more than one cell provides an incomplete picture on individual variations of physiological state, biomarker distribution or gene expression profile. With single-cell gene expression profiling one can study heterogeneity among and within cell types. However, analyzing limited number of transcripts with the most often used tecnology, QRT-PCR reaches its limit, when less than 2-fold expression changes should be determined. Therefore, although one can reliably infer qualitative trends from these gene expression results, it is difficult if not impossible to generate quantitative transcriptional data of single cells. The introduction of systematic errors in determining the exact number of RNA molecules with traditional QRT-PCR can be circumvented by using digital PCR. To demonstrate the utility, reproducibility and sensitivity of our dPCR protocol, the expression of a house-keeping gene (*rps18*), two possible cell-type specific marker genes (*npy* and *gabrd*) and three miRNA genes (miR-7, miR-132, miR-204) were recorded by dPCR from individual cellular aspirates from three different neuron types: pyramid cells (Pc), fast-spiking interneurons (Fs) and neurogliaform cells (Ngf). To confirm dPCR data for mRNA and miRNA expression of single neurons after patch-clamp recording, classical single cell QRT-PCR was also run in case of all genes on all the three neuron types used in this study. According to our data we found no altrerations in the expression of *rps18* gene. Expression of *gabrd* was specific to neurogliaform cells, significantly lower expression could be detected in fast spiking cells, and no amplification could be recorded in case of pyramidal cells.

To understand the role of miRNAs in different neuronal processes, including pathological alterations, there must be an understanding of how different miRNAs express in different cell types. The copy number of *miR-7* and *miR-132* molecules was determined in neurogliaform, pyramidal cells and in fast-spiking neurons and found to be equally expressed in all cells. We found that the expression of *miR-204* was induced in neurogliaform cells, we measured significantly lower expression in pyramidal cells.

In summary, the combination of patch-clamp recording and high-density nanocapillary dPCR protocols is an effective and sensitive approach for determining mRNA or miRNA expression in single neurons at a single molecule resolution. With the presented method one can identify individual genes participating in the establishment and maintenance of particular neuronal phenotypes, deconvolve different neuronal cell types and discover the exact distribution or variability of gene expression profiles of the electrophysiologically phenotyped cells more precisely than classical single cell QRT-PCR could achieve.

PUBLICATIONS

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