Correlations between the expression of genes encoding enzymes in the Krebs cycle and control of DNA replication in human cells

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Duplication of genetic material is a key biological process carried out in all living organisms. In eukaryotic cells, DNA replication occurs during the S phase of interphase. During this process, the double stranded DNA is unraveled due to the presence of helicase that breaks the hydrogen bonds between the DNA strands. Stability of the chromosome is ensured by topoisomerase, which modulates the spatial structure of DNA molecules. The helicase-activated primase synthesizes short (11+/-1 nucleotides) fragments of the RNA (primers), using DNA strands as templates. DNA polymerase synthesizes new strands, complementary to both original ones. When the DNA extension is completed, the DNA nuclease removes the primers, the DNA ligase links the Okazaki fragments by catalyzing the formation of phosphodiester bond. DNA polymerase fills the gaps arising after primers' removal. Errors that are not removed during replication often result in abnormal cellular functions that can lead to cancer in humans.

Central carbon metabolism (CCM) is a series of enzymatic reactions that provide the energy necessary for the development and reproduction of cells. The CCM pathways include: glycolysis, Krebs cycle, gluconeogenesis, and pentose phosphate pathways. The key role of the central carbon metabolism is the generation of energy, mainly in the form of ATP (a total of 12 molecules from one glucose molecule) and reducing agents. The Krebs cycle, also known as the citric acid cycle or the tricarboxylic acid cycle, is a series of chemical reactions aimed at generating energy by oxidation of acetate to carbon dioxide and providing reducing agents. In eukaryotes, the citric acid cycle is carried out in the matrix of mitochondria. The substrate of the cycle is acetyl coenzyme A (acetyl-CoA, active acetate), which gives citrate, when combined with oxaloacetate (coenzyme A is detached), that is subsequently oxidized to two carbon dioxide molecules as a result of isomerization, dehydrogenation, hydration, dehydration, and decarboxylation reactions. At the same time, the oxaloacetate molecule is regenerated. Additionally, 3 molecules of NAD^+ and one molecule of FAD^+ and GTP (guanosine triphosphate, ATP equivalent) are formed as a result of oxidation of one acetate residue.

Recent studies on the bacterial model of *Bacillus subtilis* indicated the existence of a direct relationship between the central carbon metabolism and DNA replication regulation. It has been observed that the temperature-sensitivity of replication mutants can be suppressed by introduction of additional mutations in the glycolytic enzyme genes. This fact is also confirmed by studies on another bacterial model, *Escherichia coli*. Mutations in the genes encoding phosphate acetyltransferase (*ata*) and acetate kinase (*ackA*), enzymes involved in the pyruvate metabolism pathway, suppressed the temperature-sensitivity phenotype caused by the presence of mutations in the *dnaA*, *dnaG* and *dnaN* genes. In addition, deletion of genes encoding glucose-phosphate isomerase (*pgi*) and phosphate acetyltransferase (*pta*) suppressed the negative effects of mutations in the *dnaB* gene (**1**).

Previous studies indicated that the involvement of CCM enzymes in the regulation of the replication process may also occur in eukaryotic organisms (**1**). It is known that some of the metabolic enzymes (e.g. *LDH* (lactate dehydrogenase), *HK* (hexokinase), *PFK* (phosphofructokinase), *GAPDH* (3-phosphoglyceric aldehyde dehydrogenase), *ALDO* (aldolase)) are present in the cell nucleus. In addition, studies conducted with ovarian adenocarcinoma cells have shown that deletion of the citrate synthase gene (*CS*) caused deregulation of cellular metabolism and decreased proliferation efficiency. Other studies carried out on other tumor cells have shown that a decrease in the expression of the *SDHA* gene (coding for succinate dehydrogenase) slowed down the rate of proliferation. In turn, the deletion of *FH* (coding for fumarase) and *SDHA/B*(encoding succinate dehydrogenase) genes in HeLa cells led to accumulation of fumarate and succinate, which eventually blocked the cell cycle.

Previous studies on DNA replication and central carbon metabolism have been carried out mostly on prokaryotic model sand tumor cells, the latter characterized by many changes in the functioning of metabolic pathways and the replication process. Therefore, the objective of my research was to determine the effect of the impaired efficiency of the expression of metabolic genes on the processes of DNA replication and repair in healthy cells derived from the human body. This criterion have been met by fibroblasts of the HDFa (Human Dermal Fibroblasts adult) line. In addition to well-functioning basic processes, these cells actively divide throughout human life. In my research, I have chosen the Krebs cycle as part of the carbon metabolism which disturbance may affect the regulation of DNA replication. In the next steps of my work, in order to assess the effects of silencing of the expression of metabolic genes on the cell cycle, I have selected those coding for following enzymes:

aconitase 2 (the *ACO2* gene), citrate synthase 1 (the *CS1* gene), isocitrate dehydrogenase 2 (the *IDH2* gene), isocitrate dehydrogenase 3 (the *IDH3B* gene), α-ketoglutarate dehydrogenase (the *OGDH* gene), succinate thiokinase 2 (the *SUCLG2* gene), succinate dehydrogenase (the *SDHA* gene), fumarase (the *FH* gene), malate dehydrogenase 1 (the *MDH1* gene), and malate dehydrogenase 2 (the *MDH2* gene). Gene expression was silenced individually using small interfering RNAs (siRNAs), which by combining with the complementary fragment of mRNA of a given gene cause the silencing of its expression. The level of mRNA after transfection (introduction of siRNA) was analyzed using reverse transcription and quantitative polymerase chain reaction (RealTimePCR or qPCR). The level of gene expression was decreased about 80-90%, with a few exceptions: *IDH3B* - 40%, *OGDH* - 60%, *SDHA* - 70%. To investigate cytotoxic effects of siRNA and the transfection reagent, I used the MTT test. Moreover, I have analyzed the incorporation of bromodeoxyuridine (BrdU) into the newly synthesized DNA, and determined cell viability using a flow cytometer with the "Count and viability" software. All the analyzes confirmed a lack of cytotoxic effects of used siRNA sand the transfection reagent on HDFa cells.

The next step of my work was the analysis of the cell cycle using the MUSE® flow cytometer with the "Cell cycle" software. I determined the percentage of cells in various phases of the mitotic division: G1, S, G2, and M. In the cells with silenced *CS1, ACO2, SUCLG, SDHA, FH, MDH2*genes,I observed about 1-4h delay of the cell entry into the S phase of the cell cycle. In addition, the silencing of the *CS1, ACO2, FH* and *MDH2* genes resulted in a lower efficiency of the DNA replication process by about 15-40% relative to the control (**2**). In addition, analyzes performed with the use of BrdU confirmed the effects of silencing of genes encoding the Krebs cycle enzymes on slowing down the cell replication rate.

In the next part of my work, I tested the effects of silencing of expression of genescoding for DNA replication and repair proteins on the cell cycle in HDFa cells. During the preliminary tests, 8 genes encoding the enzymes involved in replication were checked: *CDC6* (a gene coding for the cell division cycle protein 6), *DNA2* (the helicase/nuclease 2 gene), *HELQ* (the helicase-coding gene), *LIG1* (a gene for ligase 1), *LIG4* (a gene encoding ligase 4), TOP3B (a gene coding for topoisomerase 3β), *POLI* (a gene coding for DNA polymerase iota), *PRIM2* (a gene coding for primase 2) (I have carried out these studies together with Karolina Fornalewicz). After determining the impact of silencing of the expression of the replication and repair genes on the cell cycle, 4 genes were selected

for further work: *POLI, LIG4, TOP3B*and *PRIM*. The tests were carried out analogously to the experiments for the metabolic genes. The level of gene expression was decreased about 80-90%. Analyzes using the MTT, BrdU and flow cytometry tests ruled out any cytotoxic effect of siRNA. Importantly, the silencing of the expression of selected genes coding for proteins involved in DNA replication and repair resulted in a less efficient entry of cells into the S phase of the cell cycle and a reduced level of DNA synthesis relative to the control (**3**).

In the light of above results, I asked another question: does silencing of the expression of genes encoding Krebs cycle enzymes influence the effects of silencing of expression of replication and repair genes? To answer this question, I transfected cells with two different siRNAs (specific for a metabolic and a replication/repair gene). The level of silencing was tested by Real-time PCR. Obtained results confirmed that double silencing was as effective as in the case of silencing of a single gene. Cytotoxicity analysis confirmed the absence of a negative effect of a double amount of siRNA on HDFα cells. Thus, I carried out the analysis of the cell cycle in a similar way to previous studies, using a flow cytometer. Silencing the expression of the *POLI* gene, encoding the DNA polymerase iota, resulted in a significant reduction in the number of cells in the S phase, relative to the cells untreated with siRNAs. Additional silencing of the gene encoding one of the Krebs cycle enzymes (except *ACO2*) suppressed the negative effects of silencing of the*POLI* gene. In the case of *IDH3*, an increase in the number of cells in the S-phase above that determined for non-treated cells was observed. I also noted similar effects during double silencing of the *LIG4* gene and genes: *IDH2, IDH3B, OGDH, SUCLG, MDH1* and *MDH2*, as well as *PRIM* and *IDH2*. The silencing of *IDH2* expression also suppressed the negative effects caused by a decrease in the efficiency of the*TOP3B* gene expression. In addition, there was a group of combinations in which I did not observe any significant effects of silencing of the expression of genes encoding Krebs cycle enzymes on the changes caused by reduced expression of genes related to DNA replication and repair; in some cases even their worsening was evident (**3**). The involvement of some Krebs cycle enzymes in the DNA replication has also been demonstrated by the results of studies on the synthesis of DNA, using BrdU. What is very important, in all the cases analyzed by me, the additional silencing of one of the metabolic genes significantly increased the level of newly formed DNA in comparison to cells treated with siRNA against replication genes (**3**).

In conclusion, the results presented in my work indicated that cell cycle disturbance induced by silencing of expression of a gene involved in DNA replication/repair can be partially or even completely abolished by silencing the expression of genes encoding individual enzymes involved in the Krebs cycle. Considering the specificity of the observed suppression, the above described results can be interpreted as another genetic proof for the close relationship between DNA replication/repair and CCM in human cells. However, at the current stage of research and in the light of the available literature, I can only assume that perhaps due to the presence of some of the metabolic enzymes in the cell nucleus, the role of some of them is directly related to the replication process, such as pyruvate kinase, responsible for direct histone H3 phosphorylation. On the other hand, the observed changes may be due to the unbalanced level of metabolites and the resulting disturbances in the proper functioning of metabolic processes. Nevertheless, regardless of the molecular mechanism responsible for the effects I observed, it can be concluded that the link between DNA replication/repair and central carbon metabolism is a common biological phenomenon, present even in such far-flung evolutionary organisms as bacteria and humans.

Literature:

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