"New Aspects of Mucopolysaccharidosis Pathogenesis – Molecular Mechanisms of Cell Cycle and Apoptosis Alterations"
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Mucopolysaccharidoses (MPS) belong to the group of lysosomal storage diseases (LSD) characterized by the abnormal accumulation of lysosomal enzyme substrates (Tomatsu et al., 2018). In MPS, glycosaminoglycans (GAG) are stored due to mutations in genes encoding acidic hydrolases within lysosomes, resulting in inefficient or absent enzymatic activity. This leads to lysosomal dysfunction, disrupting cellular metabolism and causing abnormalities in tissues and organs (Wraith, 2013). Currently, around 70 LSDs, including 13 types and subtypes of MPS, have been diagnosed, with symptoms primarily affecting the skeletal, digestive, circulatory, connective tissue, and nervous systems, worsening with age (Tomatsu et al., 2018).

Until recently, the main pathogenic mechanism of MPS was believed to be the physical cell overload with undegraded GAG (Wraith, 2013). However, current therapeutic attempts, such as enzyme replacement therapy, gene therapy, hematopoietic stem cell transplantation, and substrate synthesis reduction therapy, do not fully eliminate all symptoms, even when normalizing GAG levels (Penon-Portmann, Blair, and Harmatz, 2023). Moreover, the significant symptoms variability among different MPS types indicates a more complex pathomechanism.

Recent studies suggest that GAG accumulation is only the initial stage of a cascade of changes, and secondary disorders significantly contribute to disease progression (Fecarotta et al., 2020; Leal et al., 2023). Those studies proposed that lysosomes act as regulatory centers in cellular metabolism, and dysfunctional, overloaded lysosomes may disrupt cellular processes, potentially driving MPS symptoms (Lamming and Bar-Peled, 2019; Uribe-Carretero et al., 2024). However, existing information is fragmentary, focusing on individual MPS types or subtypes and lacking insights into the molecular mechanisms of the disease.

In this context, the aim of my doctoral thesis was to investigate the molecular mechanisms of cell cycle and apoptosis alterations in all types and subtypes of MPS, specifically:

a) examine the expression levels of genes involved in the cell cycle and apoptosis,
b) determine the molecular mechanisms of cell cycle and apoptosis changes,
c) investigate the impact of genistein (a potential drug) and enzyme therapy on cell cycle alterations.

I conducted my research using a cellular model of fibroblasts derived from MPS patients and a control HDFa cell line from a healthy individual. At the time of starting my doctoral research, 11 types and subtypes of MPS were known: I, II, IIIA, IIIB, IIIC, IIID, IVA, IVB, VI, VII, and IX, and these were the subjects of the studies described in this work.

The thesis comprises three articles, published in international scientific journals, where I am the first author, forming a coherent series dedicated to cell cycle and apoptosis disruptions in MPS. The articles are as follows (presented below in chronological order relative to the date of publication, but later in the dissertation they are arranged in such a way as to constitute a substantively coherent scientific thread):

1. Brokowska J, Pierzynowska K, Gaffke L, Rintz E, Węgrzyn G (2021). Expression of genes involved in apoptosis is dysregulated in mucopolysaccharidoses as revealed by pilot transcriptomic analyses. *Cell Biol Int*. 45: 549-557. doi: 10.1002/cbin.11332.

2. Brokowska J, Gaffke L, Pierzynowska K, Cyske Z, Węgrzyn G (2022). Cell cycle disturbances in mucopolysaccharidoses: Transcriptomic and experimental studies on cellular models. *Exp Biol Med*. 247: 1639-1649. doi: 10.1177/15353702221114872.
3. Brokowska J, Gaffke L, Pierzynowska K, Węgrzyn G (2023). Enhanced efficiency of the basal and induced apoptosis process in mucopolysaccharidosis IVA and IVB human fibroblasts. *Int J Mol Sci*. 24: 14119. doi: 10.3390/ijms241814119.

Transcriptomic analysis, using RNA-seq, revealed altered expression of genes involved in the cell cycle in all examined MPS types, with the most changes observed in MPS IX (137 transcripts), MPS IIID (116 transcripts), and MPS VII (105 transcripts). Importantly, a consistent pattern was observed, where the level of each transcript was disturbed in a similar manner (either increased or decreased) in all or nearly all MPS types. These results suggest the existence of a common mechanism affecting transcriptional control or post-transcriptional processes in virtually all MPS types. Flow cytometry analysis confirmed cell cycle disturbances in most MPS cell lines, with an increased fraction of cells in the G0/G1 phase (except for MPS IVB and MPS VII) and a decreased percentage in the G2/M phase. Interestingly, the analysis of key cyclins' levels in MPS cells showed increased levels of cyclin D1, which physiologically rises in the G1 and G2 phases, in most MPS types compared to control cells. Furthermore, not only the level but also the timing of cyclin D1 overexpression was improper in MPS cells. Strikingly, transcriptomic analysis did not show significant changes in the transcript levels of the CCND1 gene, which encodes cyclin D1, indicating that other stages of the gene expression process might be disrupted.

I also investigated whether reducing GAG levels could restore proper cell cycle progression. Recombinant enzymes used clinically in MPS I (α-L-iduronidase) and MPS II (iduronate sulfatase) and genistein—a natural isoflavone tested in experimental GAG synthesis reduction therapy—were employed for this purpose. I observed that both genistein and the tested enzymes significantly improved cell cycle regulation in practically all investigated MPS cell lines. However, some aberrations were still visible, especially in MPS I cells treated with α-L-iduronidase and MPS IIIA, IIIB, IIID, VII, and IX cells treated with genistein.

Previous studies on the cell cycle in MPS only covered a few publications focusing on individual MPS types (I, II, and VII) (Moskot et al., 2016; Jiang et al., 2020; Węsierska et al., 2022). Therefore, my research is the first to comprehensively demonstrate cell cycle disturbances in MPS, both at the gene expression and molecular levels.

In terms of apoptosis, the gene expression analysis revealed altered expression of many genes related to programmed cell death in cells of all MPS types. Depending on the MPS type, the number of these genes ranged from 19 (MPS VI) to 73 (MPS IVB). The levels of most transcripts were similarly disturbed in most MPS types, suggesting common mechanisms disrupting the apoptosis process. However, the significant difference in the number of transcripts altered in terms of the expression level between MPS IV subtypes is noteworthy: 23 in MPS IVA and 73 in IVB. I conducted more detailed studies with these two cell lines, analyzing apoptosis following staurosporine stimulation. Staurosporine effectively induced apoptosis in both control and Morquio disease (MPS IV) patient-derived cells, as indicated by elevated levels of specifically cleaved caspases and PARP. Interestingly, caspase-9 levels (cleaved at Asp330) were higher in control cells than in Morquio fibroblasts, while caspase-9 levels (cleaved at Asp315) remained similar in all examined cell lines treated with staurosporine. As a result of apoptosis induction, levels of caspase-3 (cleaved at Asp175), caspase-6 (cleaved at Asp162), caspase-7 (cleaved at Asp198), and PARP (cleaved at Asp214) were higher in MPS IVA and IVB than in HDFa. Importantly, in non-induced cells, levels of caspase-3

(cleaved at Asp175) and PARP (cleaved at Asp214) were increased in MPS IVA but not in MPS IVB, relative to control cells.

My results confirmed an enhanced apoptosis in MPS IV and suggested that the execution phase is a crucial stage in this process, characterized by higher efficiency in MPS IVA and IVB cells than in control cells. This activation occurred under standard laboratory conditions (at the baseline level of apoptosis) and after staurosporine induction. Therefore, increased apoptosis in Morquio disease likely occurs at the execution phase of caspases and PARP, whose levels are elevated. This phenomenon may contribute to the pathomechanism of the disease, especially during the formation of bones, cartilage, and connective tissue.

In summary, the results of my doctoral research demonstrated disturbances in the cell cycle and apoptosis as a newly discovered mechanism of pathogenesis in all types and subtypes of MPS. Identifying the mechanisms of disruptions in cellular processes points to potential therapeutic targets and may contribute to the development of therapies that address not only the primary cause of the disease but also secondary changes. Therefore, combined therapy, involving both reducing GAG accumulation and negative regulation of cyclin D1 and/or lowering caspase levels, may be considered. It is worth noting that various cyclin D1 inhibitors are used as potential anticancer drugs (González-Ruiz et al., 2020), which could be relevant in developing new therapeutic strategies for MPS.

Among all genetic diseases, those caused by mutations in single genes seem to have a straightforward pathogenic mechanism. However, a mutation in one gene and the dysfunction or absence of one protein result not only in the inhibition of a single biochemical reaction but also in indirect complex changes, arising from the network of transactions between individual cellular processes. Therefore, studies on the molecular mechanisms of genetically-based diseases are not only necessary to understand the pathogenesis of a given disease, but are also crucial for the effective design of therapeutic methods in the future.

References:

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