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Research Article
Development of Experimental *in vitro* Models to Elucidate Molecular Mechanisms Underlying Pathology and its Prevention

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Abstract

Background and Objective: Pathological processes at the cellular level can cause damage and understanding their underlining molecular mechanisms is crucial. The study aims to develop experimental *in vitro* models to investigate these mechanisms and identify potential strategies for preventing pathology. **Materials and Methods:** The average titers of gangliosides and anti-ganglioside antibodies in samples of non-malignant mouse embryonic cells 3T3 fibroblasts, of mouse malignant myeloma cells and mixed of the two cell types *in vitro*-cultures were assessed as homogenates of each one of the three types. The tested *in vitro*-cultures were applied in the role of experimental *in vitro*-models of patients with multifactor diseases in different phases, as well as of a healthy human organism. **Results:** The assessed varieties in the average titers of the gangliosides and anti-ganglioside antibodies could be due to participation of these molecules in different in various intra- and extra-cellular inter-molecular interactions. Production of antibodies/immunoglobulins by non-lymphoid and non-hematopoietic types of cells in appropriate conditions, but also about differentiation of non-immune and non-hematopoietic cell progenitors to myeloid and lymphoid directions in appropriate conditions, were proposed. **Conclusion:** Activation of internal protective mechanisms was suggested. Further studies should be performed.

Key words: Pathological changes, prevention of disease development, initial protective mechanisms, mitochondria

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Biological effects of electromagnetic fields on intra- and extra-cellular environment have been discussed and different theories and hypotheses have been proposed[1,2]. The role of neurotransmitters, neuropeptides and many associated receptor-initiated signaling pathways with their participation in the development and progression of malignancies, but on the other hand, in their prevention and response to therapy, has been suggested[3]. The same authors have also demonstrated a cross-talk between neoplastic cells and nerve fibers as a survival strategy. Additionally, possibilities of neurotransmitters to communicate with components of other cellular types such as immune cells in the tumor microenvironment (TME), thus influencing or preventing the malignancy development, have been established[4]. Molecular mechanisms, providing interplay between the nervous and immune systems have recently been proposed, including their regulation functions on the malignancy[5]. Interplay between neurotransmitters and neuromodulators[6], but also between neurotransmitters and immunomodulators[7] has also been proven. The abnormal secretion of neurotransmitters has shown a close relationship with neoplastic progression, which has proposed unexpected breakthroughs in anti-malignancy therapy[8]. New directions concerning the role of neurotransmitters in the normal neural, but also the glioblastoma microenvironments and thus, possibilities about development of potential targeted anti-malignancy therapeutic approaches have been proven[9]. Gangliosides have been suggested to perform regulatory functions in the control of key physiological processes as cell growth and proliferation[10].

Mitochondria have been determined as primary intra-cellular sites of oxygen consumption, but also as main source of reactive oxygen species (ROS) formation[11]. Most of the ROS have been proven to originate from the mitochondrial respiratory chain[12]. Many death-inducing stimuli have been established to interact with mitochondria, thus leading to oxidative stress (OS). On the other hand, many pathological conditions have been characterized with a significantly decreased levels of the reduced Glutathione (GSH) in mitochondria[13]. Additionally, mitochondrial transplantation has been suggested as an advanced and promising therapeutic strateg[14]. As important directions of future therapeutic strategies has been suggested treatment and/or prevention of diseases caused by OS, but also against oncogenic mitochondria[15]. The abnormal signal transduction mechanisms of inter- and intra-cellular mitochondrial communications have recently been proposed as key in the aging and age-related diseases, as well as in the neoplastic transformation changes[16]. Mitochondria-dependent signaling has also been proposed to regulate both innate and adaptive immune responses[17]. Among the main intra-cellular protective mechanisms have been proven Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas systems[18]. In the last years, the attention is directed to the relationships of the RNA-alterations to different transcriptional factors activities and thus to their influence on the functions of respective genes, coding proteins, participating in various metabolic pathways[8].

In this relation, the main goal of the current study was directed to deeper investigation on the inter-molecular interactions and regulatory mechanisms, which could underline the development of multi-factor disease or in opposite, to prevent it. On cell level as underlining these disorders are proven malignant transformations on the one hand and the degenerative changes on the other. So, experimental *in vitro*-models of normal health and development of multifactor disease in people were developed by application of laboratory-incubated cultures of non-malignant mouse embryonic cells, of mouse malignant cells and mixed culture of both cell types. Molecular and light microscopy analyses were performed.

MATERIALS AND METHODS

Duration of study: The described studies were performed in the period 2022-2025.

***In vitro*-cell cultures:** Monolayer *in vitro*-cultures of 3T3 fibroblasts, derived from embryos of Balb/c experimental mice, were *in vitro*-incubated in DMEM (Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS-Sigma-Aldrich), 100 IU/mL penicillin (Sigma-Aldrich) and 100 µg/ml streptomycin (Sigma-Aldrich). At the same time, *in vitro*-cultures of transfected by recombinant DNA-plasmid mouse malignant myeloma cells P3-X63-Ag8[19], were incubated as suspension culture in growth medium RPMI 1640, supplemented with 5% FBS, as well as a mixture of 100 IU/mL penicillin (Sigma-Aldrich) and 100 µg/mL streptomycin (Sigma-Aldrich). Mixed *in vitro*-cultures were prepared by co-cultivation of cells, belonging to each one of the two types,

described above. In the current study, after formation of cell monolayer, sub-population of mouse embryonic 3T3 cells was pre-incubated in the describe above cultural medium for monolayer cultures, supplemented with cultural fluid from previously incubated in it plasmid-transfected mouse malignant myeloma cells P3-X63-Ag8, after previous centrifugation and filtration. All cell cultures were cultivated at 37°C with 5% CO_2 and 95% humidity.

Preparation of light microscopy slides: Samples from culture of the two monolayer *in vitro*-cultures (of 3T3 mouse embryonic cells and mixed by their pre-incubation) were fixed with Ethanol (Sigma-Aldrich), subsequently washed with PBS (Sigma-Aldrich) and stained by Hematoxylin and Eosin (H&E) (Sigma-Aldrich) technique. The prepared slides were analyzed by light microscope, supplemented with CCD camera.

Preparation of homogenates from the tested *in vitro* cultures: Total extracts from the laboratory-incubated cell culture of 3T3 mouse embryonic fibroblasts, from plasmid-transfected mouse malignant myeloma cells P3-X63-Ag8[19], as well as from the mixed culture of pre-incubated in supplemented media mouse 3T3 cells (described above), were prepared by mechanical homogenization, followed by treatment with 10% Trichloroacetic Acid (Cl_3CCOOH) and 0.48 M solution of K_3PO_4 and subsequent centrifugation at 3000 x for 10 min.

ELISA: The prepared homogenates of the three cell cultures were subjected on Enzyme-Linked Immunosorbent Assay (ELISA) to assess the average titers of gangliosides and of anti-ganglioside antibodies in them[20]. The received values were expressed as Mean±Standard Deviation (SD). The differences were considered as statistically significant in $p<0.05$ and $p<0.01$. The optical density (OD) was read spectrometrically at 490 nm on ELISA-reader (TECAN TM, Sunrise, Austria).

Assessment on the influence of bulk and single-cell RNA-molecules on the gene expression of *in vitro*-cultures: The influence of various bulk and single-cell RNAs on the expression of the different genes and mRNA-transcripts, coding proteins, participating in various intra- and extra-cellular inter-molecular interactions, was studies. These influences should be understood in *in vitro*-cultures of mouse embryonic 3T3 fibroblast and mixed culture of pre-incubated sub-population of 3T3 cells in cultural fluid from mouse malignant myeloma cells, were studied. So, transcriptome libraries and appropriate computer programs are prepared and applied in the European Molecular Biology Laboratory (EMBL), Heidelberg, Germany. Computer programs about determination the influence were applied.

RESULTS

Initial myeloid-like and lymphoid-like cells were observed in the fixed light microscopy slide of mixed culture (Fig. 1a). These features were expressed mainly as changed size and shape of the cells, as well as of their nuclei, which are larger and oval, but also with changed ration between the cytoplasm and nuclei. Appearance of cytoplasmic granules and vacuoles was also assessed. Thus, many cytoplasmic organelles as mitochondria, lysosomes, etc., are probably affected. Such changes in cells were not seen in the fixed preparation of the control culture of mouse embryonic 3T3 fibroblasts (Fig. 1b). The noted differences between the two *in vitro*-cultures suggested a possibility about derivation of myeloid-like and lymphoid-like cells from non-immune and non-hematopoietic cellular progenitors in appropriate conditions (as presence of malignant cells/antigens, of infectious agents/antigens, of appropriate immunomodulators, etc.). The observed changes could be explained with the existence of capable to differentiate to various directions sub-populations of stem-like cells in the general embryonic cell line.

Only in the extract form the *in vitro*-culture of mouse embryonic 3T3 fibroblasts were assessed significantly higher average titers of the anti-ganglioside antibodies compared to the average titers of gangliosides (Fig. 2a). In the homogenate form the *in vitro*-culture of mouse malignant myeloma cells P3-X63-Ag8 only at dilution 1:100 was noted higher average titer of gangliosides than of the anti-ganglioside antibodies, but the difference was not statistically significant (Fig. 2b). At the other dilutions of the same sample (2:40, 1:200 and 1:400) the average titers of the anti-ganglioside antibodies were higher than these of the gangliosides, but only at dilutions 1:200 and 1:400 the differences were statistically significant. In the extract form the mixed culture, at dilutions 1:100 and 1:300 was observed higher average titer of the anti-ganglioside antibodies compared to these of the gangliosides, but at dilutions 1:40 and 1:200 was noted the opposite tendencies (Fig. 2c). However, statistically significant differences were seen at dilutions 1:40 and 1:400, but not at dilutions 1:100 and 1:200.

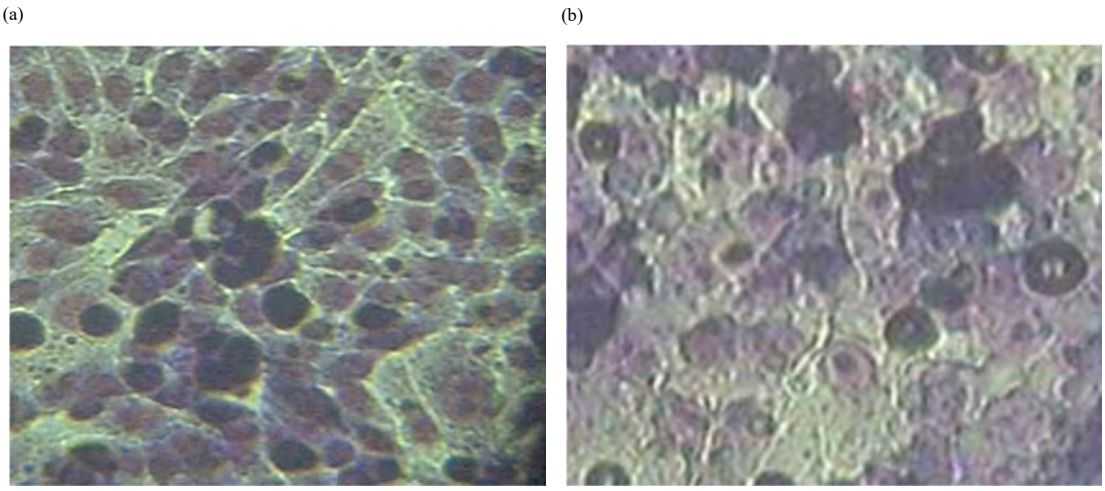

Fig. 1(a-b): Experimental *in vitro* models of mouse 3T3 cell cultures under treated and control conditions, (a) Light microscopy image of 3T3 cells pre-incubated with filtered culture supernatant from recombinant DNA-transfected P3-X63-Ag8 myeloma cells, showing emergence of early myeloid-like and lymphoid-like progenitor-like cells, suggesting possible differentiation changes within stem-like subpopulations of the culture, (b) Control 3T3 fibroblast culture showing normal morphology without treatment effects, Both images are stained with H&E and observed at ×200 magnification

H&E = Hematoxylin and eosin, ×200 = Magnification, (a) Treated group, (b) Untreated control

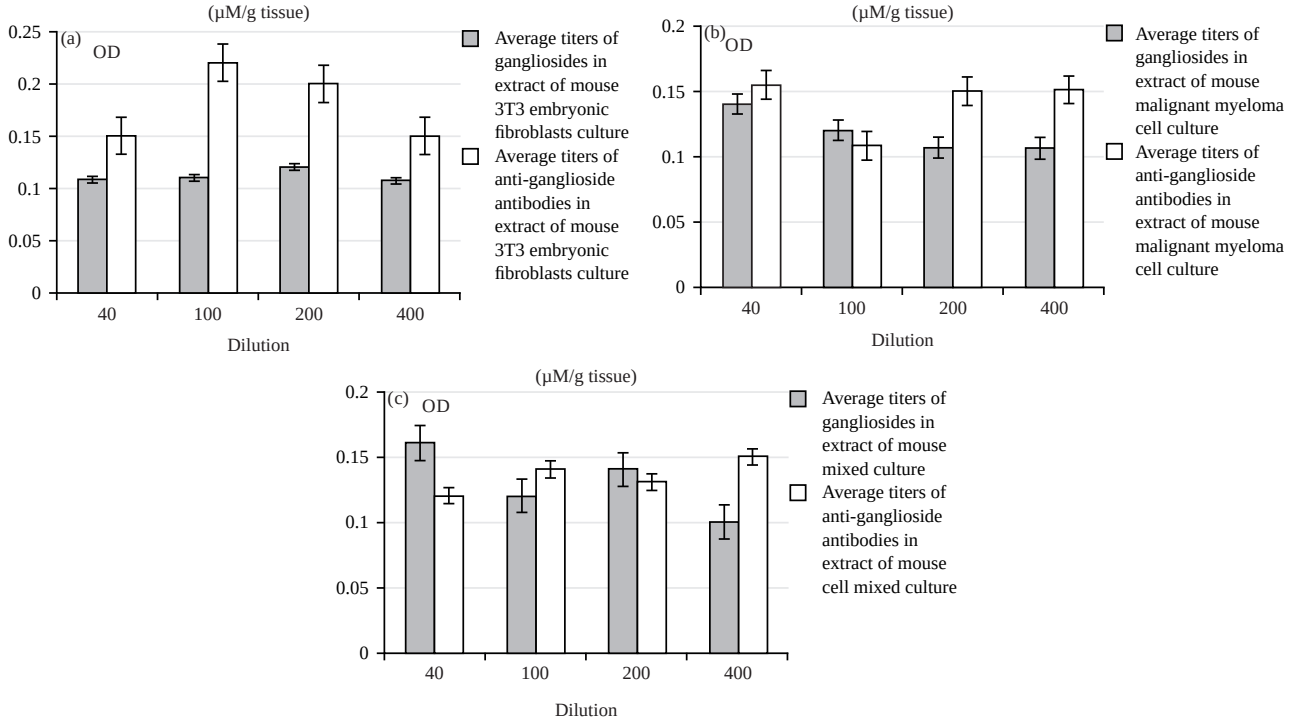

Fig. 2(a-c): Average titers of gangliosides and of anti-ganglioside antibodies in containing molecules with affinity to GSH: (a) extract of mouse *in vitro*-culture of embryonic fibroblasts from 3T3 cell line, (b) extract of *in vitro*-culture of mouse malignant myeloma cells P3-X63-Ag8, transfected with recombinant DNA-plasmid and (c) extract of mixed *in vitro*-culture of mouse embryonic 3T3 fibroblasts and mouse malignant myeloma cells P3-X63-Ag8, transfected with recombinant DNA-plasmid

Y-axis: Conc

DISCUSSION

The proposed possibility about differentiation of non-hematopoietic and non-immune cell progenitors to myeloid and lymphoid lineages was in confirmation of literature findings, related with pathways, underlining the differentiation of immature cells to myeloid and lymphoid directions in similar conditions[21,22]. The role of mitochondria in the immune regulatory mechanisms has been discussed, including in the anti-neoplastic immunity[23]. CRISPR-Cas systems have been characterized among the effective approaches about editing of the mitochondrial (mt) DNA[8]. Besides the known importance of CRISPR-Cas9 system in the nuclear DNA editing to generate mutations, which could correct specific abnormal alleles, its capability to edit the mtDNA by specific guide RNAs (sgRNAs) by targeting specific loci of the mitochondrial genome, has also been established[24]. In this way, these systems have been proposed to influence mammalian immune responses through targeting endogenous genes[25]. In this way, the link between the nucleoskeleton and cytoskeleton in the transmission of mechanical forces from the cytoplasm to the nucleus, directly affecting chromatin compactization and organization, has been underlined as particularly important[26]. As a result, activation of the persistent immune/protective mechanisms has been proposed, despite the lack of typical pathological changes. As a main determinant in the disease process spreading the communication between the altered cells and surrounding/neighboring cells, but also mitotic arrest, innate immunity and altered extra-cellular matrix[27]. Additionally, cross-reactions of the antibodies with other biological molecules were also proposed by taking in consideration respective literature findings[28]. The presented results confirmed the proven in the literature capability of many non-lymphoid cell types, including by malignant cells, to produce antibodies (immunoglobulins) in appropriate conditions[29-32]. The current data also confirmed the proven expression and production of other immune molecules (as for instance, membrane receptor glycoproteins) by non-immune and non-hematopoietic cell types[33], probably as an initial intra-cellular protective mechanism[34,35]. Activation of innate immune (protective) systems in low-differentiated cells in appropriate environment conditions have been proposed[36]. These findings have suggested the development of novel tools about annotation of single-cell data, but also about integration and interpretation of multimodal datasets covering transcriptomics, epigenomics and proteomics, as well as influencing various cellular communication networks[37,38]. In this way could be tested the different mechanisms, which could underline the development of pathology or in opposite, its prevention. The understanding, following and control of these processes is of key importance in particular in the multifactor diseases, to which below malignancies, cardiovascular, psychiatric and neurodegenerative disorders. In this way have been revealed possibilities about new insight on the immunotherapy of malignancies by understanding of specific pathway enrichment, cell communication and transcription factors, providing protection against degenerative changes at the same time[39]. These approaches can be used also to be saved information from large-scale training sets, which could be used by researchers who do not have access to large and diverse data bases[40].

CONCLUSION

The main accent of the current study was related with a better understanding of biochemical and structural changes underlining the malignant and/or degenerative changes in the cell, or, in opposite, their prevention. About a better understanding of these mechanisms on intra- and extra-cellular levels were developed experimental *in vitro* models of normal health and of developing multifactor disease, both in humans. For this purpose several different types of cell cultures with mouse origin: of non-malignant cells; of malignant cells, as well as mixed by pre-incubation of non-malignant cells sub-population in cultural fluid from previously incubated malignant cells in it. A possibility about derivation of initial myeloid-like and lymphoid-like cells from non-immune and non-hematopoietic cell progenitors in appropriate conditions (as for instance presence of malignant cells/antigens, of infectious agents or antigens, of immunomodulators, etc.) is shown. Taking in consideration the literature data, activation of initial internal protective mechanisms was suggested. In this way, regulatory processes could be activated, which could prevent malignant transformations, but also degenerative changes at the same time. Such investigations could be of key importance particularly in multifactor disorders as malignancies, cardiovascular and neurodegenerative diseases. The detection of these features could allow the establishment of the pathological process at initial stages, before appearance of the typical pathological features.

SIGNIFICANCE STATEMENT

This study presents experimentally developed *in vitro* cellular models that simulate both normal physiological conditions and multifactor disease states, providing a controlled platform to investigate molecular and intercellular mechanisms underlying pathology. By integrating

non-malignant, malignant and mixed cell culture systems, the work highlights potential cellular transitions, immune-like responses and ganglioside, antibody dynamics that may reflect early regulatory or protective mechanisms in disease development. The findings contribute to a better understanding of cell-to-cell communication, immune modulation and mitochondrial-associated processes, offering a foundation for future studies aimed at early detection, prevention strategies and therapeutic targeting of multifactor diseases including malignancies and degenerative disorders.

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