ejpmr, 2017,4(04), 34-38

SJIF Impact Factor 4.161

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

DEVELOPMENT OF STRATEGIES FOR PREVENTION OF NEOPLASTIC PROCESSES ON CELLULAR AND ORGANISM LEVELS IN VITRO AND IN VIVO. A PILOT STUDY

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Article Received on 27/01/2017 **Article Revised on 17/02/2017** **Article Accepted on 10/03/2017**

ABSTRACT

Different methods for prevention of neo-plastic transformation and activation of anti-malignant properties of *in vitro*-cultivated cells on both cellular and organism levels for *in vitro*- and *in vivo*-applications were developed and tested. Commercial mixed recombinant DNA-constructs, based on adeno-associated virus (AAV) (Parvoviridae family) DNA-genome were designed, containing copy of oncogene and of tumor-suppressor gene, respectively. The observed signs of early myeloid and phagocytic differentiation of non-transfected normal embryonic cells, co-cultivated with different types of transfected and non-transfected cells, containing malignant antigens, was accepted as a proof for preserved normal/non-malignant characteristics of the transfected cells, containing additional oncogene copy, on the one hand, but also for a strategy for support of adequate anti-malignant immune response, on the other, mainly by the protein products of the oncogenes in their role of active antigens. As a confirmation about the anti-neoplastic activity of tumor-suppressor gene was the noticed improvement in the general blood picture of experimental Balb/c mice, inoculated with both malignant cells and transfected mESCs, containing additionally-inserted copy of this gene, in comparison with that of animals, inoculated only with malignant cells.

KEYWORDS: Recombinant DNA-constructs, gene transfection, cell co-cultivation.

INTRODUCTION

The high self-renewal potential of the stem cells makes them strong candidates for delivering of genes, as well as for restoring organ systems function have been found to be included in these processes.[1-4] This understanding could be applied toward the ultimate goal of using stem cells for various therapeutic goals, but also as a tool to discover the mechanisms, underlining the support of balanced activity between the different genes and coded by them protein products, both in vitro and in vivo.[3-5] Studies on them are most often directed to investigation on the mechanisms of their self-renewal and differentiation.[1-4, 6-8] The properties of "malignant stem cells", have outlined initial therapeutic strategies against them.[4-9] On the other hand, a rapid lymphoid-restricted (T-, B- and NK) reconstitution capacity in both in vitro- and in vivo-conditions has been reported in stem cells from bone marrow material of adult laboratory mice.[10]

A lot of messages for gene transfer in cells could be performed by viruses with DNA-[11, 12] and/or RNA-genome,[13, 14]. Similar results have also been achieved by application of mixed recombinant constructs, designed on the basis of bacteria plasmid DNA[15] or of yeast[16] DNA-genomes.

In this connection, the main goal of the current study was directed to development and investigation of methods for prevention of malignant transformation in laboratory-incubated cells.

MATERIALS AND METHODS

Development of methods for maximally safe insertion of oncogenes and tumor-suppressor genes in normal mouse embryonic stem cells by transfection with appropriate recombinant viral gene constructs

Previously designed commercial recombinant gene constructs were used by application of protocol, described by Chen et al., 2003, which were based on adeno-associated virus DNA, containing promoter of Eukaryotic Elongation Factor-1 alpha (EF-1α) gene from adeno-associated virus *(*Parvoviridae family);[11] copy of the respective gene of interest (oncogene Dcn1 and tumor-suppressor gene HACE-1, respectively), as well as marker gene, determining resistance to Neomycine, isolated from bacteria plasmid DNA, received by treatment with appropriate restrictases and subsequently – with ligases. Gene, determining antibiotic resistance, was necessary for the selection of positive and negative on the respective gene of interest transfected normal and malignant cells, and it was located in immediate nearness to the last. For this goal, all transfected cells were incubated in the presence of the synthetic analogue of

Neomycine – G418. Normal stem cells, isolated from mouse Balb/c embryos (mouse embryonic stem cells - mESCs), were incubated for 48 - 72 hours on previously formed monolayers of feeder layer of mouse embryonic fibroblasts after previous treatment by Mitomycin-c (mm-c) (Sigma-Aldrich), after which they are tripsinized and consequently transfected by electroporation (5 x 10^6 cells/ml). The possibility of the used recombinant DNA-constructs and transfected by them positive on additional copy of mouse gene HACE-1 cells was provided by insertion of tag for human FLAG protein. On the 8^{th} to the 10^{th} day after the transfection, selection of cell clones from both transfected types, resistant to Neomycin was made by their cultivation in medium, containing diluted substance G-418, known as a synthetic analogue of Neomycin. Around the 28^{-th} day after the seeding in the presence of G418 (Sigma-Aldrich), more of the so cultivated cells died, and only those, which contained the gene, connected with resistance to Neomycine, survived and proliferated.

Assessment of presence or absence and expression activity of additionally-inserted copies of the tested genes of interest in transfected normal mESCs

All types of the selected and proliferated normal mESCs were subjected on genomic assay, by isolation of total DNA and RNA from all transfected and non-transfected sub-populations of both cell types, followed by standard Polymerase Chain Reaction (PCR) and Reverse Transcriptase PCR (RT-PCR), with application of appropriate 3'- and 5'-DNA-primers, complementary to the used recombinant gene constructs, with subsequent electrophorhesis in 1% Agarose gel, containing previously added Ethidium bromide solution.

Prove of the in vivo-usability of transfected mESCs, containing additionally-inserted tumor-suppressor gene copy, on appropriate experimental rodents

Experimental Balb/c mice were used and separated in five groups: inoculated with suspension of transfected normal mESCs, containing additionally-inserted copy of tumor-oncogene Dcn1; inoculated with suspension of transfected normal mESCs, containing additionally-inserted copy of tumor-suppressor gene HACE-1; with suspension of transfected malignant human cervical carcinoma HeLa cells; with suspension of malignant HeLa cells and subsequently – with suspension of transfected mESCs, containing additional copy of tumor-suppressor gene HACE-1, and control group of non-inoculated animals. The cell suspensions were received by treatment of the respective cell monolayer with solution of trypsin/EDTA (Sigma-Aldrich) and Trypan Blue Dye Exclusion Test, with subsequent centrifugation and resuspendation in PBS (Sigma-Aldrich). 28 days after the cell inoculation, all mice were sacrificed. Fixed light-microscopy preparations were prepared from peripheral blood from the tail vein, by fixation of blood smears with 95% Ethanol (Sigma-Aldrich), washing with PBS (Sigma-Aldrich), staining with Hematoxilin/Eosin (Sigma-Aldrich), subsequent washing and drying at room temperature. The prepared slides were observed by inverted light microscope, supplied with mega-pixel CCD-camera.

All animal procedures were performed in accordance with Animal Ethics Committee.

Assessment of the in vitro-anti-malignant phagocytic properties of myeloid progenitors, derived from normal mouse embryonic cells, co-cultivated with mouse malignant cells

Mixed cultures of normal cells from mouse embryos, co-cultivated with mouse malignant cells, were prepared, by both incubation of the normal cells in cultural fluid from malignant cells (after previous filtration and centrifugation), as well as in the presence of the cultural fluid plus malignant cells. All cultures were incubated in Dulbecco's Modified Minimal Essential Medium (DMEM) (Sigma-Aldrich), supplemented with 10% Fetal Calf Serum (FCS) (Sigma-Aldrich), 100 U/ml Penicillin (Sigma-Aldrich) and 100µg/ml Streptomycin (Sigma-Aldrich), at 37^0C in incubator with 5% CO_2 and 95% air humidification. Fixed light-microscopy preparations were prepared by fixation with 95% Ethanol, washing with PBS, and staining with Giemsa (Sigma-Aldrich), with subsequent washing and drying at room temperature. The prepared slides were observed by inverted light microscope, supplied with mega-pixel CCD-camera. Separate sub-populations of non-transfected normal mESCs were co-cultivated with of transfected mESCs with proved presence of additional oncogene copy, other non-transfected sub-group – in the presence of malignant cells and the result were compared with a respective control cellular sub-population. All cells were incubated at 37^0C in incubator with 5% CO_2 and 95% air humidification, in Dulbecco's Modified Minimal Essential Medium (DMEM) (Sigma-Aldrich), supplemented with 10% Fetal Calf Serum (FCS) (Sigma-Aldrich), 100 U/ml Penicillin (Sigma-Aldrich) and 100 µg/ml Streptomycin (Sigma-Aldrich). For confirmation of changes in the cells, indicated by 3D-imagination, laser irradiation and combination of both methods, but also of the reliability of these techniques, scanning elctrone microscopy (SEM) analysis of the same probes are necessary in the future. Remove "For this goal, after turning-off of the cultural fluids, the cells were washed twice with PBS and 2.5% glutaraldehyde. The so prepared probes were observed by scanning electrone microscope Philips 515.

RESULTS

The presence of additional copy of oncogene Dcn1 and tumor-suppressor gene HACE-1 in separate ESCs sub-populations was proved by PCR analysis (Figure 1 - a), but the expression of both genes in the same cell sub-groups was also indicated by the results of agarose gel electrophorhesis after previous RT-PCR assay (Figure 1 - b).

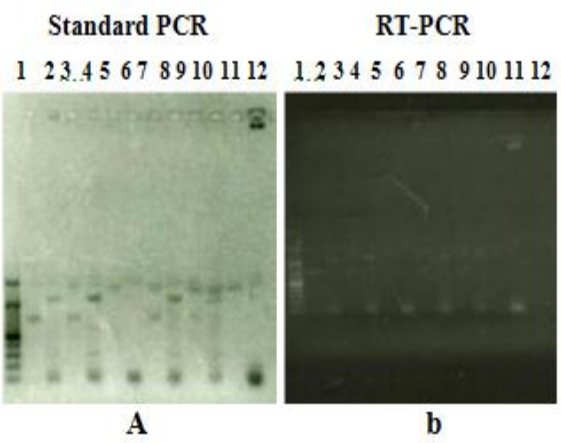
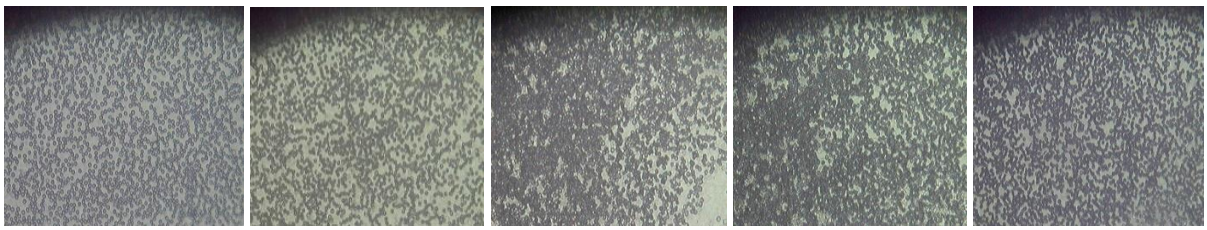


Figure 1: 1% agarose gel electrophorhesis of genome DNA from normal mESCs after performed standard PCR (a) and RT-PCR (b), for prove the presence or absence of additional copies of oncogene Dcn1 (lines 3; 5; 9) and tumor-suppressor gene HACE-1 (lines 2; 4; 8), inserted by transfection with recombinant adeno-associated viral DNA-construct, containing copy of the respective gene of interest, designed previously by application of specific primers, complementary to the used recombinant DNA; lines 6; 7; 10; 11; 12 – total genome DNA-material from normal mESCs without additional gene copy; line 1 – marker (M)

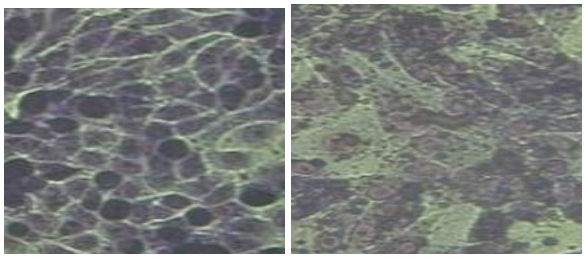
No significant differences in the general blood picture of the experimental mice, inoculated with transfected mESCs, containing additional copy of both oncogene Dcn1 (Figure 2 - b), as well as of tumor-suppressor gene HACE-1 (Figure 2 - e) in comparison with that of the control animals (Figure 2 - a), were noted. Also, an improvement in the blood picture of rodents, inoculated with both malignant HeLa cells and subsequently – with transfected mESCs, containing additional tumor-suppressor gene copy (Figure 2 - d), compared with the observed in animals, inoculated only with the malignant cells (Figure 2 - c), was observed.



a b c d e

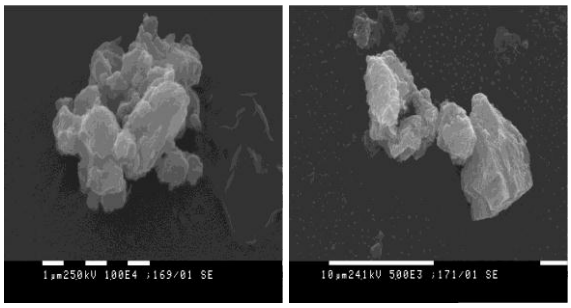
Figure 2: Peripheral blood smears from Balb/c experimental adult mice: a) from non-inoculated control animals; b) from mice, inoculated with suspension of transfected mESCs, containing additional copy of oncogene Dcn1; c) from mice, inoculated with suspension of malignant HeLa cells from human cervical carcinoma; d) from mice, inoculated with suspension of malignant HeLa cells and subsequently – with transfected mESCs, containing additional copy of tumor-suppressor gene HACE-1; e) from mice, inoculated with suspension of transfected transfected mESCs, containing additionally-inserted copy of tumor-suppressor gene HACE-1 (Hematoxillin/Eosin staining, magnification: x100)

In co-cultivation of normal mouse embryonic cells in the presence of mouse malignant cells, characteristics of early myeloid differentiation, as nuclei/cytoplasm ratio and appearance of cytoplasmic granules (Figure 3), as well as cytoplasmic excrescences and phagocytic properties (Figure 4), were noted.



a b

Figure 3: In vitro-cultivated normal mouse embryonic cells: a) control embryonic cell culture; b) mixed cell culture of normal mouse embryonic cells and malignant mouse cells (Giemsa-staining, magnification: x100)



a b

Figure 4: SEM photos of preparations: a: control - clusters of normal mouse embryonic cells could be seen; b: mixed culture of normal embryonic and malignant myeloma cells – two large cells, each one in process of malignant cell phagocytosis, could be observed

DISCUSSION

The observed myeloid and phagocyte changes in non-transfected normal mESCs, co-cultivated with non-

transfected malignant cells, were accepted as proof for preserved normal/non-malignant character of the last, on the one hand, as well as for support of adequate anti-malignancy immunity, on the other, mainly by the protein product of the oncogene in its role of active antigen. In many tumor tissues and cultivated cell lines, active expression of oncogene DCUN1D3 (Dcn1) has been assessed.[17, 18] This gene has been characterized as a regulator of gene p53. Gene Dcn1 has also been found to be sufficient for cullin neddylation in a purified recombinant system, as well as, on the other hand – contribution of its over-expression to malignant disorders, as well as a potential marker for metastatic progression.[19,20] Links between DNA-replication, chromatin and proteolysis has been confirmed by proved cullin-RING E3-ubiquitin ligases, assembled on the CUL4 platform.[19] The observed signs of myeloid and phagocyte cell differentiation in co-cultivation with malignant cells, are in support of the literature sources about the influence of appropriate factors on the differentiation direction.[21]

In opposite to oncogene Dcn1, tumor-suppressor gene HACE-1 codes protein-kinase, which, in opposite to kinase protein product of gene Dcn1, makes easier targets for degradation key proteins of the malignancy and metastasis processes.[17, 20, 22] The anti-malignancy activity of this gene was confirmed by the noted improvement in the general blood picture of experimental Balb/c mice, subsequently inoculated with malignant cells HeLa and transfected mESCs, containing additionally-inserted copy of it, in comparison with the data of rodents, inoculated only with malignant HeLa cells.

CONCLUSION

Methods for prevention of malignant processes in activation of two genes with contrary functions are suggested, by development of strategies with appropriate combinations of other internal (genetic), epigenetic and external factors.

ACKNOWLEDGEMENTS:

We are grateful to Assoc. Prof. Biliana Georgieva, PhD, Institute of Optical Materials and Technologies "Acad. J. Malinowski", Bulgarian Academy of Sciences, and to Assoc. Prof. Daniela Karashanova, PhD, Institute of Optical Materials and Technologies "Acad. J. Malinowski", Bulgarian Academy of Sciences, for the help in the Scanned Electronic Microscopy (SEM) observations performance.

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