

Development of Methods for Safe Application of Viral Vectors for Gene-Engineering Manipulations

Sainova IV^{1*}, Valkova IP², Markova TZ³ and Nikolova EB¹

¹Bulgarian Academy of Sciences, Institute of Experimental Morphology, Pathology and Anthropology with Museum, "Acad. Georgi Bonchev" Street, Bl. 25, 1113 Sofia, Bulgaria

²1st Pediatric Consultative Clinic, Sofia, Bulgaria

³Department of Pharmacology and Toxicology, Medical University, BG – 1000 Sofia, Bulgaria

*Corresponding author: Iskra Ventseslavova Sainova, Bulgarian Academy of Sciences, Institute of Experimental Morphology, Pathology and Anthropology with Museum, "Acad. Georgi Bonchev" Street, Bl. 25, 1113 Sofia, Bulgaria, Tel: +359-884761224; E-mail: iskrasainova@gmail.com

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Abstract

Additional oncogene copy was transferred in normal cells, and additional tumor-suppression gene copy in malignant cells, respectively, by transfection with the appropriate recombinant DNA-constructs, containing each one the respective gene. The results obtained were compared with the data from insertion of copy from the respective gene in prokaryotic bacteria strains *E. coli*, by analogical transfection with appropriate gene constructs. In application of low initial infections (high initial dilutions of viral suspensions, respectively) of eventually attenuated by many passages vaccine strains from the heterologous for mammals and mammalian cells avian viral species, only slight ultra-structural changes and signs of partially-formed immature viral particles in the cells, were established, but no cytopathic effect (CPE) was observed. In freezing of the so inoculated mammalian cells after addition of cryoprotector Dimethylsulfoxide (DMSO), thawing and re-incubation in fresh cultivation medium, signs of activated cell proliferation were noted. One of the possible explanations could be eventual transfer of nucleotide sequences from viral particles to separate cells because of activated fusion processes on the influence of DMSO in drastic temperature changes. The results obtained proposed a possibility about application of the described methods as available alternatives for both vaccine production and gene-engineering manipulations with cells, compared with the procedures of preparation and application of designed quite expensive recombinant DNA-constructs. Future studies are necessary in this direction.

Keywords: Mammalian cells; Vaccine viral strains; Nucleotide transfer; Activated fusion process

Introduction

Many literature data about the role of viruses as appropriate vectors for gene transfer have been obtained for both viruses, which genome is DNA [1-5] and/or RNA [6-7] have been proved. Messages about application of mixed recombinant DNA-constructs, composed by DNA-fragments have also been obtained containing components from different viral strains [8-10] or from different organism origin (viral, bacteria plasmid, yeast and/or other eukaryotic cell types) [11-12]. For this goal, appropriate promoter gene should be inserted, necessary to provide the expression of the respective inserted gene of interest. The other main component is a marker gene, localized to immediate nearness to the tested gene of interest, necessary for selection of the cells, positive of the last. Most often, as marker genes could be used such, which code color proteins (for example, fluorescent protein, as GFP, YFP, RFP) or products, participating in a color reaction (enzymes luciferase or timidinkinase), or is connected in any way resistance to any antibiotic preparation. The described gene sequences should be connected by treatment with specific ligases, but the used initial vector constructs should contain specific restriction sites, which is necessary the withed DNA-fragments of interest be obtained by treatment with respective restriction enzymes (most often bacterial endo-nucleases). Other important components are the reverted end DNA-repeats. A lot of messages about the role of cryo-protector Dimethylsulfoxide

(DMSO) and other organic detergents, for activation of the fusion of the cell, as well as between separated cells, have been received [13,14].

In this connection, the main goal of the current study was connected with development and investigation of methods for maximally safe application of viral strains as appropriate vectors for gene transfer in mammalian species.

Materials and Methods

Development of methods for maximally safe insertion of genes in mammalian cells by designed recombinant viral gene constructs

Additional copy of oncogene was inserted in mESCs, as well as of tumor-suppressor gene – in malignant cells, respectively, by previously-designed recombinant DNA-constructs, containing appropriate promoter; copy of the respective gene of interest, as well as marker gene, determining antibiotic resistance, 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 positively and negative on the respective gene of interest transfected normal and malignant cells, and it was located in immediate nearness to the last. 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 resuspension in PBS (Sigma-Aldrich).

Assessment of presence or absence and expression activity of additionally-inserted gene copies in normal and malignant mammalian cells

All types of the selected and proliferated eukaryotic mammalian normal cells, as well as prokaryotic bacteria strains, both transfected and non-transfected, as well as containing and non-containing additional copy from each one of the respective genes tested, 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 electrophoresis in 1% Agarose gel, containing previously added Ethidium bromide solution. Total protein material, eventually containing the respective tumor-suppressor protein of interest, was isolated malignant rat insulinoma RIN-5F mammalian cells, but also from prokaryotic *E. coli* bacteria strains, both transfected with respective appropriate recombinant DNA-vectors, containing copy of rat tumor-suppressor gene. Protein lysates from non-transfected eukaryotic and prokaryotic cells from both types were analogically prepared and tested. The so isolated protein extracts were subjected on separation by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), in the presence of specific mono-clone antibody against the tumor-suppressor protein, marked by FITC.

Development of techniques preparation of viral vectors, applicable for maximally safe gene-engineering manipulations with mammalian cells

Methods for safe applications of avipoxviral strains FK (fowl) and Dessau (chicken) (Poxviridae family) as initial material for design of appropriate vectors for immunization and gene-engineering manipulations with mammals and mammalian cells, were developed. Cells of embryonic bovine trachea were used. Cell line from embryonic bovine trachea, derived by Kniazeff et al. [15], was used. Three main steps for development of strategy about safe application of both viral strains were evaluated: application of heterologous for mammals and cells from them avian viral strains; of attenuated vaccine forms of both strains, as well as of comparatively low initial infectious titers of 10^3 CCID50/ml viral suspension (high initial dilutions of viral suspensions of 10^{-3} CCID50/ml, respectively). Separated sub-populations of mammalian cells, inoculated with 3 lgs lower initial infectious titers of 10^6 CCID50/ml viral suspension (with 3 lgs higher initial dilutions of viral suspension 10^6 CCID50/ml, respectively), of both vaccine viral strains, were frozen at -80°C or at -196°C (liquid nitrogen) after previous addition of respective volume from the cryo-protector Dimethylsulfoxide (DMSO) (Sigma), added directly to the inoculated cell cultures, for 1-2 weeks. After thawing, the so prepared cultures were re-cultivated at 37°C , in incubator with 5% CO_2 and 95% air humidification. For this aim, all thawed cell suspensions were centrifuged, and after turning-off of the supernatants, the pellets were washed with PBS (Sigma), resuspended, and the cell suspensions received were put in fresh cultivation medium, composed of the gradients, described above.

Preparation of light-microscopy slides

After turning-off the cultural fluids, the cells were washed with PBS (Sigma) and fixed with 95% Ethanol (Sigma). After washing with PBS (Sigma), the preparations were stained by Hematoxylin/Eosin (Sigma), after which they were washed and dried at room temperature. The so prepared slides were observed by inverted light microscope, supplied with mega-pixel CCD-camera. Cell cultures, subjected on freezing and subsequent thawing, were analogically observed as native light-microscopy preparations.

Preparation of fixed slides for transmission electron microscopy (TEM) observation

After turning-off the cultural fluids, the cells were washed twice with PBS (Sigma) and fixed in 10% Fomaline. The so prepared probes were put on nets on drops from the obtained liquid suspensions. For ultrastructural assay, TEM – JEOL JEM2100 microscope, with maximal tension of 200 kV and magnification $200 \div 1\,500\,000\text{ X}$ was applied.

Results

Besides the presence of additional oncogene copy in separate normal cell sub-populations, proved after PCR assay, its expression activity in the same cell sub-groups was also indicated by the results of agarose gel electrophoresis after previous RT-PCR analysis (Figure 1).

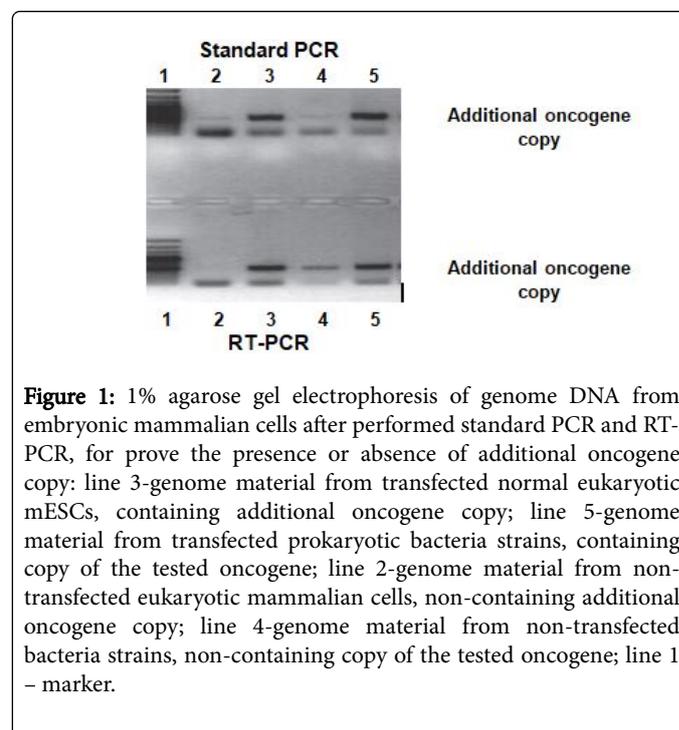


Figure 1: 1% agarose gel electrophoresis of genome DNA from embryonic mammalian cells after performed standard PCR and RT-PCR, for prove the presence or absence of additional oncogene copy: line 3-genome material from transfected normal eukaryotic mESCs, containing additional oncogene copy; line 5-genome material from transfected prokaryotic bacteria strains, containing copy of the tested oncogene; line 2-genome material from non-transfected eukaryotic mammalian cells, non-containing additional oncogene copy; line 4-genome material from non-transfected bacteria strains, non-containing copy of the tested oncogene; line 1 – marker.

Similarly, in transfected malignant cells and prokaryotic *E. coli* bacteria strain, containing additionally-inserted copy of rat tumor-suppressor gene, not only the presence of the last, but also its active expression was proved by SDS-PAGE assay of the isolated protein material (Figure 2).

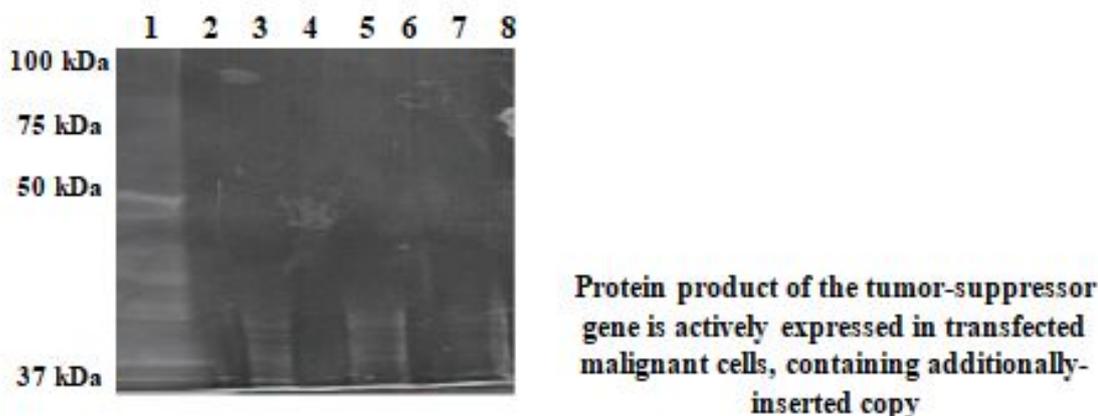


Figure 2: SDS-Polyacrilamide gel electrophoresis of protein material, isolated from eukaryotic malignant RIN-5F rat insulinoma cells (line 3) and prokaryotic *E. coli* bacteria strains (line 5), both containing additionally-inserted copy of rat tumor-suppressor gene by transfection with previously constructed recombinant viral DNA-constructs, containing it; line 1 – marker; line 2 – protein material, isolated from non-containing additional tumor-suppressor gene copy non-transfected eukaryotic malignant RIN-5F rat insulinoma cells; line 4-non-containing tumor-suppressor gene copy non-transfected bacteria cells *E. coli*; lines 7 and 8 – isolated rat tumor-suppressor gene protein product – 10-fold diluted (line 7) and undiluted (line 8).

Prove of viral presence in infected cells, and determination of appropriate ways about safe gene-engineering applications

In the cell cultures, inoculated with initial dilutions of initial infections titers of 10^3 CCID₅₀/ml (high initial dilutions of viral

suspensions of 10^{-3} CCID₅₀/ml) of both strains (Figures 3B and 3C), for the first time cytopathic effect (CPE) was not observed by light microscopy assay and no significant differences in comparison with the control non-infected cells (Figure 3A) were established.

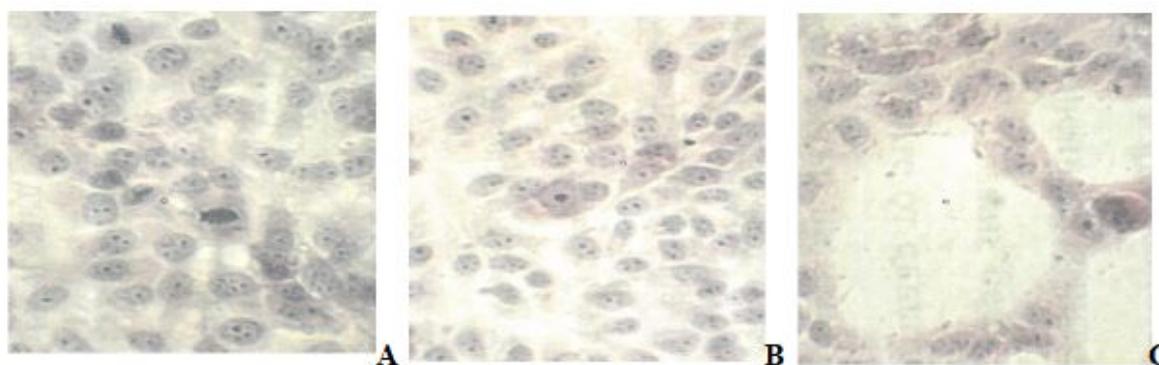


Figure 3: Fixed light-microscopy preparations of normal mammalian embryonic cells: A non-inoculated control – actively proliferating, several dividing cells in various stages of the mitotic cycle could be seen; B-inoculated with vaccine pigeon avipoxviral strain Dessau (pigeon); C-inoculated with fowl pox viral strain FK (stained by Giemsa dye, magnification: x200).

Investigation of ultra-structural differences between non-infected and infected with low initial infectious titers of both tested vaccine avipoxviral strains

In observation of the probes, described above transmission, by electron microscopy (TEM), some ultra-structural differences were

noted in the inoculated cells in comparison with the non-inoculated (Figure 4), performed mainly by changes in the cytoplasmic organelles structure and order, as well as formation of immature virion-like particles (Figures 4B and 4C). In the probe, infected with the fowl pox viral strain FK, formation of lots viral inclusions and immature viral particles was observed (Figure 4C). In the cell culture, inoculated with

the pigeon strain Dessau, approximately 2-3 immature viral particles in the process of their leaving the cell were noted (Figure 4B), the formation of viral inclusions was slight, the cytoplasmic structure was

nearer to this of the control non-inoculated cells (Figure 4A) than in the cells, inoculated with the fowl vaccine strain.

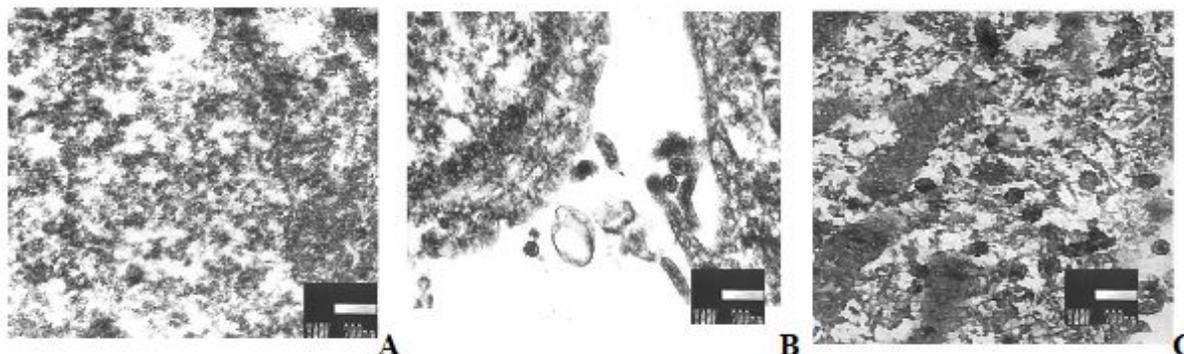


Figure 4: TEM photos of normal mammalian embryonic cells: non-inoculated with both tested vaccine avipoxviral strains, with initial infectious titers: A-non-inoculated control mammalian cells; B – inoculated with the vaccine fowl pox viral strain FK; C – inoculated with the vaccine pigeon pox viral strain Dessau (magnification: x40000).

Development of cell fusion techniques with mammalian cells, inoculated with low initial infectious titers of heterologous for them vaccine viral strains

In application of 3 lgs lower initial infectious titer of 10^6 CCID50/ml (of 3 lgs higher initial viral suspension dilutions of 10^{-6} CCID50/ml, respectively) for both vaccine viral strains, not only no cyto-pathogenic changes were observed, but signs activated cell proliferation in both cases in comparison with the non-inoculated control were noted

(Figure 5), as increased cell density, decreased pH value of the cultural fluid (yellowish staining of the native preparations – Figures 5B and 5C, compared with the native control – Figure 5A) and formation of internal cellular “islands” in the monolayer and the. One of the possible explanations was eventual insertion of viral genetic material in the cellular genome of separated cells because of activated fusion process on the influence of cryo-protector DMSO in drastic temperature changes.



Figure 5: Native light-microscopy preparations of normal mammalian embryonic cells: A-non-inoculated control; B-inoculated with lower initial infectious titer of vaccine pigeon avipoxviral strain Dessau (pigeon); C-inoculated with lower initial infectious titer of fowl pox viral strain FK (magnification: x100).

Discussion and Conclusion

The proved high self-renewal potential of the stem cells in *in vitro*-conditions 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 [9,10,12,16]. This understanding could be applied toward the ultimate goal of using stem cells not just for various forms of therapy, but also as a tool to discover the mechanisms and means to bring, reconstituting them from old and young individuals

has exhibited indistinguishable progenitor activities *in vitro* and *in vivo* [12,17]. The properties of “malignant stem cells”, have outlined initial therapeutic strategies against them [12,18]. The observed improvement in the blood picture from experimental rodents, inoculated with both non-transfected malignant cells, non-containing additional tumor-suppressor gene copy, and transfected normal cells, containing additional oncogene copy, could be accept as a proof for support of adequate cellular and humoral immune response, mainly by the protein products of the oncogenes in their role as appropriate anti-

neoplastic antigens. So, the received data obtained were in agreement with the literature findings for the safety of used for transfer of nucleotide sequences in cells viral vectors [7,19]. Also, these results are in agreement with the literature data about indicated rapid lymphoid-restricted (T-, B-and NK) reconstitution capacity *in vivo* and completely lacked myeloid differentiation potential of both *in vivo* and *in vitro* in stem cells from bone-marrow material of adult laboratory mice in respective appropriate conditions [20].

The obtained data were in agreement of many literature data about proved ways for safe applications of avian viral strains on non-permissive for them mammalian cells [21]. One of the probable explanations about the limited viral replication possibilities could be the non-permissive for avian strains mammalian cells, which is in agreement with the literature data, as well as with these, obtained in our previous studies. The potential of viruses as appropriate vectors for development of various therapeutic strategies has been proved in many literature sources – for both immunization by application of recombinant vaccines [2,8,9,22] and therapeutic procedures in different disorders by substitution of appropriate cell nucleotide sequences [4,7]. The efficiency of DNA-repair has been found to vary greatly among the different cell types [16]. Besides of the possibility for application in various therapeutic strategies, these properties have been characterized as usable to discover of many inter-molecular mechanisms underlining main life processes both *in vivo* and *in vitro* [19,23,24]. In this connection, the lack of CPE in mammalian cells, inoculated with initial dilutions of 10^3 CCID50/ml (with viral suspensions dilutions of 10^{-3} CCID50/ml, respectively), but the presence of immature viral particles, could suggest a possibility about application of the strains in this form as material in vaccine production, including for the goals of the immunotherapy of malignancies. The higher amount of immature virions in cells, inoculated with the vaccine viral strain FK in comparison with this in the cells, infected with strain Dessau, is in support of our previous results about the proved stronger *in vitro*-CPE, induced by the fowl vaccine strain than the induced by the pigeon vaccine strain [25,26]. In this way, a possibility for application of the vaccine fowl pox viral strain FK as a usable source for preparation of vaccines for immunoprophylaxis and immune-therapy was supposed, also in agreement with the literature finding about the high immunogenic potential of this strain [27,22]. On the other hand, the proposed transfer of nucleotide sequences from viral particles to mammalian cells as a result of activated fusion on the influence of DMSO in drastic temperature changes was in support of the reference data about such effects of this cryo-protector [13,28], but also of other organic detergents [14]. As a main reason, underlining the fusion activation between membrane structures in these conditions, was suggested the changes in the properties of the membrane-building molecules, as well as in the inter-molecular interactions, on the influence of some organic substances [28]. This method could be applied as a more available alternative for both vaccine production and gene-engineering manipulations with cells, in comparison with the procedures of preparation and application of designed quite expensive recombinant DNA-constructs and small interference RNA-sequences (siRNAs). Further studies are necessary in this direction.

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