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Gene-Armed Oncolytic Poxvirus Against Cancer

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Abstract: A set of oncolytic vaccinia viruses (VACV) expressing different variants of imaging, immune-boosting and anti-cancer therapeutic molecules, was created. To improve safety, recombinant VACV were engineered to specifically target only cancer cells by deletion genes encoding thymidine kinase and viral growth factor. In place of these genes following artificial genes with modified codon composition optimized for expression in mammalian cells were introduced: granulocyte-macrophage colony stimulating factor (GM-CSF) for induction of tumor-specific cytotoxic T lymphocytes, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) for induction of apoptotic or non-apoptotic cell death by cross-linking with DR4 or DR5 (TRAIL receptors) and green fluorescent protein (GFP) for diagnostic use.

Key words: Cancer · Gene-Armed Oncolytic Poxvirus · GM-CSF · TRAIL · GFP

INTRODUCTION

The main trend in the treatment of cancer today –is a complex approach, the main objective of which is to enhance the effectiveness of traditional therapies with new treatments. One of these methods is the biotherapy of tumors by oncolytic viruses, the advisability of which is due to their ability in addition to direct cyto-destructive actions to affect the whole body, restoring its natural anti-cancer effects by inducing a specific and non-specific immune responses [1].

Among all the studied viruses, particular attention should be paid to the vaccinia virus (VACV) which has a tropism for a wide range of mammalian cells [2]. One of the infectious forms of the virus - EEV has an additional lipoprotein membrane, which allows the virus to escape the action of neutralizing antibodies, helping to spread to distant secondary tumors (metastases) [3]. Virus replication occurs entirely in the cytoplasm of the cell, thus eliminating any interaction with the host genome, while a long history of clinical use in the eradication of smallpox makes it safe for use in human.

The purpose of this study was to create a platform that contains a set of oncolytic vaccinia viruses expressing immune-boosting and anti-cancer therapeutic molecules. For the address lysis of tumor cells the genes encoding thymidine kinase (TK) and viral growth factor (VGF), the violation of which almost completely reduces the ability of the virus to replicate in normal cells of the body, were inactivated in the genome of the VACV [4]. In place of these genes artificial genes were embedded that encode a green fluorescent protein (GFP) for diagnostic use, protein TRAIL, that induces apoptosis along p53 independent way for many types of tumor cells [5], an immunostimulatory protein granulocyte-macrophage colony stimulating factor (GM-CSF), that reinforces expression of MHC class II antigens on human monocytes and enhances cytotoxicity of neutrophils against cancer cells [6].

MATERIALS AND METHODS

Vaccinia Virus and Cell Cultures: African green monkey kidney cells (CV-1) and human laryngeal carcinoma cells (Hep-2) were obtained from the SRC VB "Vector" collection and were grown in Dulbecco's modified Eagle's medium (DMEM, Biolot, Russia) containing 10 % fetal bovine serum (FBS, HyClone, USA) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) in an incubator at 37°C with 5 %CO_2. The stock of vaccinia virus strain LIVP used in this study for construction of all recombinant viruses was received from the SRC VB "Vector" collection.

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Table 1: Integration plasmids for construction of oncolytic vaccinia viruses

Integration plasmid	Inactivated gene	Inserted gene
pΔTK	TK	-
pΔTK/GMCSF(+)	TK	GM-CSF
pΔVGF	VGF	-
pΔVGF/TRAIL(+)	VGF	TRAIL
pΔVGF/GFP(+)	VGF	GFP

Construction of Integration Plasmids: To implement the time dominant selection method [7], a set of integration plasmids was created, each carrying a dominant selectable marker gene (gpt *E. coli* gene under the control of 7.5K VACV promoter) disposed outside of long regions of homology to virus DNA; VACV genome sequence containing and flanking the disturbed gene encoding TK or VGF; artificial polylinker sequence containing a synthetic early-late promoter [8] and termination signals of transcription and translation (TTTTTNT); gene sequences encoding GM-CSF, TRAIL and GFP (Table 1). Each construct was confirmed by restriction digestion and DNA sequencing using a 310 Genetic Analyzer (Applied Biosystems, USA). DNAs of the plasmids pΔTK, pΔTK/GMCSF(+), pΔVGF, pΔVGF/TRAIL(+) and pΔVGF/GFP(+) were prepared in bulk using an EndoFree Plasmid Mega Kit (Qiagen, USA) and suspended in sterile PBS. The DNA concentrations were determined by UV spectroscopy using an Ultrospec 3000 pro (Biochrom, UK) spectrophotometer.

Virus Cloning by Plaques Method: Confluent cells grown in six-well plates were infected with pre-sonicated and filtered through a 0.45 μm virus in 0.5 ml of DMEM without serum for 1 hour at 37°C with 5 % CO_2. After removing the growth medium with virus material and washing the cells with DMEM without serum, 1.5 ml of DMEM supplemented with 2 % FBS and 1 % low gelling temperature agarose (BioReagent, USA) was added for 48 hours at 37°C with 5 % CO_2 with following addition of 1.5 ml of DMEM supplemented with 2 % FBS, 1 % low gelling temperature agarose and neutral red and incubation for extra 5 hours at 37°C with 5 % CO_2. Resulting individual virus plaques were isolated and passaged in 24-well plates with confluent cells in 0.5 ml of DMEM supplemented with 2 % FBS for 48 hours at 37°C with 5 % CO_2 with following two freeze-thaw cycles. Aliquots from each clone were characterized for the presence of deletion/insertion by PCR analysis.

Transfection: Confluent cells grown in six-well plates in DMEM without serum and antibiotics were infected with corresponding parent virus at a multiplicity of infection of 0.1 for 1 hour at 37°C with 5 % CO_2. After removing the growth medium with virus material and washing the cells with DMEM without serum, pre-incubated for 30 minutes complex of lipofectamine (Invitrogen, USA) with 2 μg of corresponding integration plasmid DNA in 1.5 ml of DMEM supplemented with xanthine (250 μg/ml), hypoxanthine (15 μg/ml) and mycophenolic acid (25 μg/ml) without serum was added to cells and incubated for 5 hours at 37°C with 5 % CO_2 with following replacement of the medium with 1.5 ml of DMEM supplemented with xanthine (250 μg/ml), hypoxanthine (15 μg/ml), mycophenolic acid (25 μg/ml) and 2 % FBS and incubation for extra 48 hours at 37°C with 5 % CO_2.

Virus Replication in Cultered Cells *In vitro*: Confluent cells grown in six-well plates were infected with virus from transfection step or previous passage in 1.5 ml of DMEM supplemented with 2 % FBS for 48 hours at 37°C with 5 % CO_2. After two freeze-thaw cycles to lyse the cells and release virus, virus was used for next passage or cloning.

Viral DNA Extraction: Confluent CV-1 or Hep-2 cells were infected with recombinant VACV. After 48 hours when cytopathic effects were complete with following two freeze-thaw cycles, viral DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, USA), according to the manufacturer's instructions. Briefly, 200 μl of total suspension was used for DNA extraction and 100 μl of elution buffer was used for DNA elution.

PCR Analysis: The mono- or double-deletion/insertion structure of selected recombinant VACV was confirmed by PCR using the following primers:

for the J2R gene, encoding TK
5'-ATATGTTCTTCATGCCTAAACGA-3' and
5'-ATGAAGGAGCAAAAGGTTGTAAC-3';
for the C11R gene, encoding VGF
5'-AGGAAGGAGGCATGGAACTC-3' and
5'-CTGATAAAAATGGAAAAGAGTTATG-3'.

The presence of the deletion/insertion in TK or VGF region of the recombinant virus DNA was detected according to the length of the amplified fragments. The amplified fragment of the wild type TK has a length of 1875 bp; inactivated TK, 1980 bp; inactivated TK with inserted GM-CSF, 2403 bp. The amplified fragment of the wild type VGF has a length of 1746 bp; inactivated VGF, 1851 bp; inactivated VGF with inserted TRAIL, 2349 bp; inactivated VGF with inserted GFP, 2559 bp.

RESULTS AND DISCUSSION

At the first phase of work, due to the high heterogeneity of the VACV LIVP strain, it was necessary to obtain a characterized clonal variant of the virus. Cloning of the original strain of the VACV LIVP from the collection of SRC VB "Vector" was performed by plaques method on the monolayer of transplanted green monkey kidney CV-1 cell culture under solid agarose-coating [9]. 16 clonal virus variants were obtained. Analysis of selected clones was performed by PCR on the ten key genes encoding virulence factors: J2R, C11R, A56R, N1L, B8R, F4L, E3L, C3L, J2R, C12L. Also genomic regions containing and flanking regions of two target genes (J2R and C11R), encoding TK and VGF respectively, were sequenced. For further work a clonal variation of VACV LIVP was selected that was positive for main genes encoding virulence factors and with the nucleotide sequence of areas of the disturbed genes corresponding to VACV LIVP strain (GenBank: AY678276.1).

For obtaining recombinant viruses with inactivated genes encoding TK and VGF and inserted genes encoding GM-CSF, TRAIL and GFP, we selected the time dominant selection method [7]. For construction of integration plasmid pΔTK, intended for inactivating the VACV genome gene encoding TK, two PCR fragments of viral genome were prepared with length of 750 bp each, dividing the gene encoding TK, on the right and on the left. A DNA of the previously selected clonal VACV variant strain LIVP was used as a template. These left-flanking (L) and right-flanking (R) PCR fragments were treated with restriction enzymes and ligated to plasmid vector pMGCgpt, containing a dominant selectable marker [10]. Further, a synthetic polylinker was inserted into the resultant construction between the L and R fragments followed by confirmation of the nucleotide sequences by sequencing on an automated sequencer 310 Genetic Analyzer (Applied Biosystems, USA).

Preparation of integration plasmid pΔVGF, designed for inactivating the VACV genome gene encoding VGF was conducted similarly to the above using DNA fragments of L and R flanks of the gene encoding VGF.

During the next step the genes encoding GM-CSF and TRAIL, with modified codon composition optimized for expression in mammalian cells, were obtained from the long synthetic oligonucleotides using PCR. A gene encoding a phenotypic marker GFP was obtained by PCR using as a template plasmid pIRES2-AcGFP1 (Clontech Laboratories, USA). Next, the obtained genes were cloned into plasmids pΔTK and pΔVGF under control of synthetic early-late promoter for construction of integration plasmids pΔTK/GMCSF(+), pΔVGF/TRAIL(+) and pΔVGF/GFP(+). The correct nucleotide sequences of all integration plasmids were confirmed by sequencing.

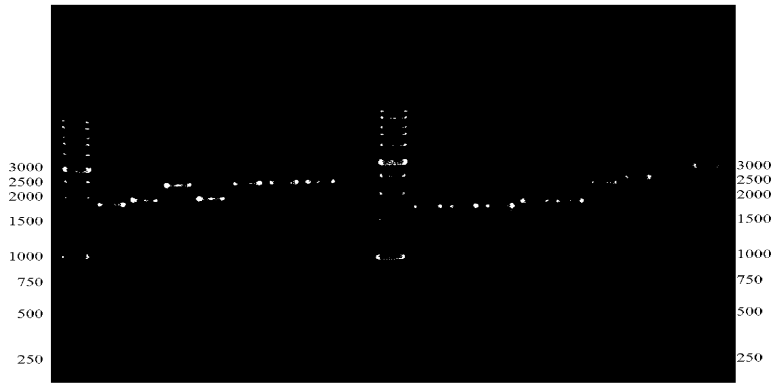

Fig. 1: Agarose gel electrophoresis of DNA fragments obtained by PCR analysis of recombinant viruses using primers for the genes encoding TK and VGF (length of PCR products is shown):

1. VACV (TK: 1875 bp, VGF: 1746 bp);
2. pVACVΔTK (TK: 1980 bp, VGF: 1746 bp);
3. pVACVΔTK/GMCSF(+) (TK: 2403 bp, VGF: 1746 bp);
4. pVACVΔTKΔVGF (TK: 1980 bp, VGF: 1851 bp);
5. pVACVΔTKΔVGF/ GMCSF(+) (TK: 2403 bp, VGF: 1851 bp);
6. pVACVΔTKΔVGF/ GMCSF(+)TRAIL(+)(TK: 2403 bp, VGF: 2349 bp);
7. pVACVΔTKΔVGF/ GMCSF(+)GFP(+)(TK: 2403 bp, VGF: 2559 bp);
8. Negative control;

M. DNA marker, length in bp shown on the left and right sides.

Table 2: Constructed recombinant oncolytic vaccinia viruses

Recombinant VACV	Inactivated gene	Inserted gene	Oncolytic potential
pVACVΔTK	TK	-	Increased tropism toward cancer cells
pVACVΔTK/GMCSF(+)	TK	GM-CSF	Increased tropism toward cancer cells, stimulation of immune response against cancer
pVACVΔTKΔVGF	TK, VGF	-	Absolute tropism toward cancer cells
pVACVΔTKΔVGF/ GMCSF(+)	TK, VGF	GM-CSF	Absolute tropism toward cancer cells, stimulation of immune response against cancer
pVACVΔTKΔVGF/ GMCSF(+)TRAIL(+)	TK, VGF	GM-CSF, TRAIL	Absolute tropism toward cancer cells, stimulation of immune response against cancer, apoptosis activation in tumor cells
pVACVΔTKΔVGF/ GMCSF(+)GFP(+)	TK, VGF	GM-CSF, GFP	Absolute tropism toward cancer cells, stimulation of immune response against cancer, detection of tumor cells

Based on the selected clonal variant of vaccinia virus strain LIVP using integration plasmids (Table 1) the recombinant VACV were obtained with deletion of the genes encoding TK and / or VGF and integrated genes encoding GM-CSF, TRAIL and GFP (Table 2). Transfection step implemented using lipofectamine (Invitrogen, USA) according to manufacturer's procedure and six subsequent passages in continuous CV-1 cells culture were conducted in selective culture medium DMEM (Biolot, Russia) with 2% fetal bovine serum (HyClone, USA) in presence of xanthine (250 μg/ml), hypoxanthine (15 μg/ml) and mycophenolic acid (25 μg/ml). After removal of selective pressure the VACV LIVP recombinant cloning was performed by plaque formation on a monolayer of CV-1 cells or human laryngeal carcinoma cells Hep-2 under a solid agarose coating [9]. The extraction of viral DNA from independent clones was performed using the QIAamp DNA Mini Kit (Qiagen, USA), followed by PCR analysis using primers specific for screening clones in genomic regions containing and flanking the target genes J2R and C11R (Figure 1). Correctness of the nucleotide sequence of the target genes encoding TK and VGF and inserted genes coding for GM-CSF, TRAIL and GFP in all selected clones of recombinant viruses were confirmed by sequencing. Efficiency of expression of GM-CSF and apoptosis-inducing protein TRAIL in created recombinant VACV has been demonstrated by Western blot analysis using polyclonal antibodies obtained to prokaryotic variants of these proteins.

CONCLUSION

The platform consisting of six oncolytic vaccinia viruses expressing phenotypic marker, immunostimulatory and anti-cancer therapeutic molecules has been created (Table 2). Inactivation of the genes encoding thymidine kinase and viral growth factor will provide high tropism of created viruses to tumor cells. Expression of GM-CSF will provide a better presentation of cancer antigens to the immune system of the organism and the expression of the apoptosis-inducing protein will correct the mutations in the genome of tumor cells and activate apoptosis. Given the high tropism, the recombinant virus expressing the fluorescent protein will be focused on very small amounts of cancer cells and fast replication of the virus will allow production of thousands of copies of a fluorescent protein in each cancer cell, which will help detect the tumor and the presence of subsequent metastasis.

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