EP 2 571 367 B1

EUROPEAN PATENT SPECIFICATION

Date of publication and mention of the grant of the patent: 13.01.2016 Bulletin 2016/02

Application number: 11783849.0

Date of filing: 04.04.2011

Int Cl.: C12N 7/00 (2006.01) A61K 35/76 (2015.01)

International application number: PCT/US2011/000602


Designated Contracting States: AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

Priority: 18.05.2010 US 800585

Date of publication of application: 27.03.2013 Bulletin 2013/13

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- WO-A1-2008/144067
- US-B2- 7 247 297

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• SPANN ET AL.: 'Genetic recombination during coinfection of two mutants of human respiratory syncytial virus.' J. VIROL. vol. 77, 2003, pages 11201 - 11211, XP008164763

• BUKREYEV ET AL.: 'Interferon gamma expressed by a recombinant respiratory syncytial virus attenuates virus replication in mice without compromising immunogenicity.' PROC NATL ACAD SCI USA vol. 96, no. 5, 1999, pages 2367 - 2372, XP002149108
FIELD OF THE INVENTION

The invention is within the scope of oncolytic virotherapy. We engineered respiratory syncytial virus (RSV) by deleting NS1 gene, and found that the NS1 gene deficient RSV (ΔNS1 RSV) can kill breast cancer cells, but not normal human cells.

BACKGROUND OF THE INVENTION

Breast cancer: Breast cancer is the most commonly cancer among women, with more than one million new cases identified worldwide each year [1]. An estimated 192,370 patients were newly diagnosed with breast cancer in the United States in 2009, and about 40,170 died of the disease [2]. Approximately 24% to 30% of women who have no lymph-node involvement at the time of diagnosis will relapse; the relapse rate for node-positive women is between 50%-60%[3]. The 5-year survival rates for those diagnosed with regional and metastatic disease are 80% and 26%, respectively[3]. Therefore, a safe and effective treatment remains a critical need.

Oncolytic virotherapy. Oncolytic virotherapy is a novel strategy using viruses, either naturally occurring or genetically modified, to selectively target and destroy tumor cells whilst leaving surrounding non-malignant cells unharmed[4]. The destruction of cancer cells occurs either through direct lytic rupture by multi-cycle viral replication or the subsequent induction of apoptosis[5] and successful application of virotherapy requires preferential and efficient amplification of the virus to lyse cancer cells. NS1 gene deficient RSV(ΔNS1 RSV) functions as an oncolytic virus against breast cancer.

RSV biology. RSV belongs to the family Paramyxoviridae, subfamily Pneumovirinae, genus Pneumovirus. The viral RNA is approximately 15 kb in size and is flanked by a leader region at the 3’ extremity of the genome and by a trailer region at the 5’ extremity (Fig. 1). The viral genome contains individual genes for ten viral proteins [6]. The NS1 gene, unique to members of the genus Pneumovirus [7], is promoter-proximally located at the 3’ end of the viral genome and its mRNA is the most abundant of the RSV transcripts in a linear start-stop-restart mode [8]. NS1 protein is referred to as nonstructural since it has not been detected in RSV particles. NS1 is exclusively found in RSV-infected cells. Our group, along with others, has found that NS1 can counter the type I IFN signaling during RSV infection [9, 10], implying that NS1 plays a direct role in inhibiting the host’s innate immune response.

RSV can be rendered nonpathogenic by mutating the NS1 gene so that it no longer inhibits IFN release, which attenuates viral infection in normal cells. However, these nonpathogenic RSV, ΔNS1 RSV, are still oncolytic because tumor cells are defective in their ability to produce and respond to IFN and, therefore, efficiently support the propagation of ΔNS1 RSV.

SUMMARY:

The present invention provides an NS1 gene-deficient oncolytic respiratory syncytial virus (RSV) for use in the treatment of breast cancer. In one embodiment, the gene NS1 is deleted by the removal of 122 to 630 nt in the antigenomic cDNA using reverse genetics approach, resulting in the joining of the upstream nontranslated region of NS1 to the translational initiation codon of NS2. The ANS1 RSV was recovered through co-transfecting Vero cells with the NS1-deficient RSV cDNA and expressional plasmids encoding N, P, M2-1 and L. The RSV NS1 protein functions as a type-I-IFN antagonist, ΔNS1 RSV virotherapy produces more type-I-IFN, which prevents virus from replication in normal cells and also induces antitumor effects.

In another embodiment, the ANS1 RSV can be applied to cancer spot by direct injection. Or the ANS1 RSV can be delivered to cancer spot through blood transfusion.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Diagram of the RSV genome and its transcription and replication products.

Fig. 2A. Verify viral NS1 protein by immunoblotting using anti-NS1 antibodies.

Fig. 2B. Morphology of virus-infected MDA-MB-231 and CCD-1059 SK cells 24h post-infection.
Fig. 2C. Viral titers as measured by plaque assay at 24h after infection. Standard deviations from three independent experiments are shown by the error bars.

Fig. 2D. In vivo test virotherapy. Subcutaneous MDA-MB-231 tumors were implanted in BALB/c nude mice and the size was photographed. Control mice received equal volume of vehicle or PBS. Tumor sizes were measured at the end of treatment. Each data point represents a mean of 6 tumors measurements plus or minus the standard deviation.

Fig. 2E. The tumor sizes were measured, and virotherapy is indicated by arrows below the x-axis. Control mice received equal volume of vehicle or PBS. Tumor sizes were measured at the end of treatment. Each data point represents a mean of 6 tumors measurements plus or minus the standard deviation.

Fig. 2F. Viral titers were measured in different tissue homogenates from the same animal to test virus safety after three days injection of viruses.

Fig. 2G. Viral F gene expression\[9\] was analyzed by RT-PCR to test virus safety in different organ from the same individual after three days virotherapy.

Table 1. Cytopathic effect (CPE) test showing ΔNS1 RSV selectively kills human breast cancer cells

DETAILED DESCRIPTION OF THE INVENTION

The respiratory syncytial virus (RSV) was used in this study. The NS1 gene was deleted by the removal of 122 to 630 nt in the antigenic cDNA using reverse genetics approach, resulting in the joining of the upstream nontranslated region of NS1 to the translational initiation codon of NS2. The ΔNS1 RSV was recovered through co-transfecting Vero cells with the NS1-deficient viral cDNA clone and expressional plasmids encoding N, P, M2-1 and L. Alternatively, the engineered virus could be any other viruses with the deletion of similar NS1 gene.

To identify whether ΔNS1 RSV lacks NS1 gene, we infected Vero cells (IFN-β gene deficient cells) with wt RSV and ΔNS1 RSV (MOI=5), NS1 protein were tested using NS1 specific antibodies by immunoblotting. As shown in Fig.2A, NS1 protein was only visualized in wt RSV-infected Vero cells, not ΔNS1 RSV-infected cells, indicating that ΔNS1 RSV lacks NS1 gene.

ΔNS1 RSV preferentially kills breast cells both in vitro and in vivo. MDA-MB-231 breast cancer cells and normal CCD-1059SK (Human normal breast fibroblast) were cultured in as indicated by ATCC (American Type Culture Collection) instruction, and then infected with wt and ΔNS1 RSV (MOI=5). Changes in cell morphology were observed and viral replication was measured. Fig. 2B shows that ΔNS1 RSV selectively induces cytopathic effect (CPE) in MDA-MB-231 breast cancer cells, and that ΔNS1 RSV has a higher viral titer in this tumor cells than in CCD-1059SK cells 24h post-infection (Fig. 2C), suggesting that MDA-MB-231 cells efficiently support the propagation of ΔNS1 RSV. To test if ΔNS1 RSV also kills other breast cancer cell lines, we infected breast cancer cell lines T-47D and MCF-7 with ΔNS1 RSV (MOI=5). CPEs were observed 48 h post-infection (Table 1), indicating ΔNS1 RSV specifically kills breast cancer cells.

To determine whether ΔNS1 RSV infection induces tumor growth regression in vivo, MDA-MB-231 breast cancer cells were injected s.c. into the left and right flanks of 4-6 weeks old nude BALB/c mice (n=6 per group) and the resulting tumors were allowed to develop. Viruses (1 X10\(^{10}\) pfu/ml) were locally injected into the tumors three times and the sizes of the tumors were measured using digital calipers. Fig. 2C, D show that ΔNS1 RSV infection caused regression in tumor growth versus controls. To test the safety of locally administered viruses, the virus titer in various organs of infected mice was determined by plaque assay and RT-PCR assay. As shown in Fig. 2E, F, the viruses specifically localize to tumors.

ΔNS1 RSV infection induces apoptosis in tumor cells, but not in normal human breast fibroblast CCD-1059SK cells. To test the differential effect of ΔNS1 RSV infection on apoptosis, MDA-MB-231 tumor cells and normal CCD-1059 SK cells were infected with the indicated viruses (MOI = 5) and apoptosis was measured by the annexin V binding assay.
Fig. 3A shows that $\Delta$NS1 RSV selectively induces apoptosis in tumor cells, compared to the cell spontaneous apoptosis shown in control.

[0016] Knockdown of the RSV NS1 gene allows the production of more IFN-β in A549 cells [9]. To further study the involvement of IFN-β in virus-induced apoptosis in breast cancer cells, neutralizing Abs against IFN-β were used to block IFN activity, but failed to attenuate apoptosis in breast cancer cells induced by viral infection (Fig. 3B). To confirm this finding, we infected Vero cells (IFN-β gene deficient cells) with $\Delta$NS1 RSV, apoptosis was measured by the annexin V binding assay. Fig. 3C shows that $\Delta$NS1 RSV still induces apoptosis in Vero cells, compared to the control, suggesting that IFN may not be involved in virus-induced apoptosis.

Table 1. $\Delta$NS1 RSV selectively kills human breast cancer cells

<table>
<thead>
<tr>
<th>Virus (MOI=10)</th>
<th>CPE (hr post-infection)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24hr</td>
</tr>
<tr>
<td>CCD-1059Sk (Human normal breast fibroblast)</td>
<td>--</td>
</tr>
<tr>
<td>MDA-MB-231 (Breast, adenocarcinoma, p53-)</td>
<td>++++</td>
</tr>
<tr>
<td>T-47D (Breast, ductal carcinoma, p53-)</td>
<td>++</td>
</tr>
<tr>
<td>MCF7 (Breast, adenocarcinoma, p53+)</td>
<td>++</td>
</tr>
</tbody>
</table>

Note: -: no CPE; ++: CPE ≤ 50%; ++++: CPE ≥75%

Reference:

[0017]


Claims

2. The NS1 gene-deficient oncolytic RSV for use according to claim 1, wherein said NS1 gene has been deleted.

3. The NS1 gene-deficient oncolytic RSV for use according to claim 2, wherein said NS1 gene has been deleted by a reverse genetics strategy.

4. The NS1 gene-deficient oncolytic RSV for use according to claim 3, wherein said NS1 gene has been deleted by the removal of 122 to 630 nt in the antigenomic cDNA.

5. The NS1 gene-deficient oncolytic RSV for use according to any preceding claim, requiring co-expression in Vero cells of five viral components from transfected plasmids, namely the NS1-deficient viral cDNA clone, viral N, M2-1, P protein and L protein.

6. The NS1 gene-deficient oncolytic RSV for use according to any preceding claim, for administration to cancer by direct injection.

7. The NS1 gene-deficient oncolytic RSV for use according to any of claims 1-5, for administration to cancer through blood transfusion.

Patentansprüche

1. NS1-Gen-defizientes onkolytisches Respiratorisches Synzytial-Virus (RSV) für die Verwendung bei der Behandlung von Brustkrebs.

2. NS1-Gen-defizientes onkolytisches RSV für die Verwendung gemäß Anspruch 1, wobei das NS1-Gen ausgeschaltet wurde.

3. NS1-Gen-defizientes onkolytisches RSV für die Verwendung gemäß Anspruch 2, wobei das NS1-Gen durch eine reverse Genetikstrategie ausgeschaltet wurde.

4. NS1-Gen-defizientes onkolytisches RSV für die Verwendung gemäß Anspruch 3, wobei das NS1-Gen durch die Entfernung von 122 bis 630 nt in der antigenomischen cDNA ausgeschaltet wurde.

5. NS1-Gen-defizientes onkolytisches RSV für die Verwendung gemäß einem vorhergehenden Anspruch, wofür Co-Expression in Vero-Zellen von fünf viralen Komponenten transfizierter Plasmide erforderlich ist, nämlich dem NS1-defizienten viralen cDNA-Clon, viralen N-, M2-1-, P-Protein und L-Protein.

6. NS1-Gen-defizientes onkolytisches RSV für die Verwendung gemäß einem vorhergehenden Anspruch zur Verabreichung für Krebs durch direkte Injektion.

7. NS1-Gen-defizientes onkolytisches RSV für die Verwendung gemäß einem der Ansprüche 1-5 zur Verabreichung für Krebs durch Bluttransfusion.

Revendications

1. Virus respiratoire syncytial (RSV) oncolytique déficient en gène NS1 destiné à être utilisé dans le traitement du cancer du sein.

2. Virus RSV oncolytique déficient en gène NS1 destiné à être utilisé selon la revendication 1, ledit gène NS1 ayant été délété.

3. Virus RSV oncolytique déficient en gène NS1 destiné à être utilisé selon la revendication 2, ledit gène NS1 ayant été délété par une technique de génétique inverse.

4. Virus RSV oncolytique déficient en gène NS1 destiné à être utilisé selon la revendication 3, ledit gène NS1 ayant été délété par suppression de 122 à 630 nucléotides (nt) dans l’ADNc antigénomique.
5. Virus RSV oncolytique déficient en gène NS1 destiné à être utilisé selon l'une quelconque des revendications précédentes, nécessitant la co-expression dans des cellules Véro de cinq composants viraux provenant de plasmides transfectés, à savoir le clone d'ADNc viral déficient en NS1, les protéines L, P, M2-1 et N virales.

6. Virus RSV oncolytique déficient en gène NS1 destiné à être utilisé selon l'une quelconque des revendications précédentes, pour administration à un cancer par injection directe.

7. Virus RSV oncolytique déficient en gène NS1 destiné à être utilisé selon l'une quelconque des revendications 1 à 5, pour administration à un cancer par transfusion sanguine.
FIG. 1. Diagram of the RSV genome and its transcription and replication products.
The virus genes are depicted as grey rectangles; the L gene, which comprises almost half of the genome, has been truncated. The GS and GE signals are shown as white and black boxes, respectively. The encoded anti-genome and mRNAs are indicated by hatched rectangles. Arrows indicate the location of the promoters.
Fig. 2E
Figure 2F

Viral titers (10^3 pfu/ml)

- Delta NS1 RSV
- wt RSV

Tumor Lung Kidney Spleen Heart
Figure 2G

Tumor  Tumor  Lung  Kidney  Spleen  Heart

wt  ΔNS1 RSV

RSV-F  GAPDH
REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- US 2004109877 A [0006]

Non-patent literature cited in the description