Research Article

rAAV2-mediated Gene Expression In Primary Cortical Neural Cells Following Inhibition Of DNA Synthesis and/or EGFR Tyrosine Kinase

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Abstract

Aim: In an effort to enhance rAAV2 transduction efficiency in primary cortical neural cells, we examined the combined effects of chemicals known to increase rAAV2 transduction efficiency in many other cell types.

Results: Unexpectedly, pretreatment of neural cells with genotoxic agents (hydroxyurea or aphidicolin) and/or the EGFR tyrosine kinase inhibitor, tyrphostin-1, did not improve transduction efficiency. SK-Hep1 cells expressing dramatic increase in rAAV2 transduction following chemical combination, experienced both significant release from G1/G0 phase and phosphorylation of p38, whereas no in neural cells.

Conclusion: Our results suggest that chemical pretreatment with hydroxyurea, aphidicolin and/or tyrphostin-1 is unable to increase rAAV2 transduction efficiency in neural cells, indicating that other strategies should be investigated.

Keywords: tyrphostin-1, primary neural cells

Primer Kortikal Nöronal Hücrelerde DNA Sentezi ve/veya EGFR Tirozin Kinaz İnhibisyonu Takiben RAAV2-e Bağlı Gen Ekspresyonu

Özet

Amaç: Primer kortikal nöronal hücrelerde rAAV2 transdüksiyon etkinliğini arttırmak üzere bu çalışmada diğer birçok hücre tiplerindeki rAAV2 transdüksiyon etkinliğini arttıran kimyasalların kombine etkilerini araştırdık.

Sonuçlar: Hidroksiüre veya afidikolin gibi genetoksik ajanlarla ve/veya EGFR tirozin kinaz inhibitörü olan triptostin-1 ile nöronal hücrelerin ön tedavisi, beklenmedik bir şekilde, transdüksiyon etkinliğini arttırdı. SK-Hep1 hücreleri kimyasal kombinasyonu izleyen rAAV2 transdüksiyonunda dramatik bir artışa neden olarak hem G1/G0 fazlarından hem de p38 fosforilasyonundan önemli ölçüde salınıma neden oldular. Nöronal hücrelerden böyle bir etki gözlemlememdi.

Yargı: Bu sonuçlar hidroksiüre, afidikodin ve/veya triptostin-1 ile kimyasal ön-tedaviyle rAAV2 transdüksiyon etkinliğinin nöronal hücrelerde arttırmalarının olanaksız olduğunu ve diğer stratejilerin araştırılması gerektiğiini göstermektedir.

Anahtar Kelimeler: rAAV2, transduction efficiency, hydroxyurea
Introduction
Recombinant adeno-associated virus vectors, derived from a dependent human parvovirus, can transduce non-dividing normal cells to yield long-term transgene expression associated with a minimal immunological response. Among these vectors, a recombinant adeno-associated virus serotype 2 vector (rAAV2) is capable of transducing neural cells, making it an important potential vector for delivery of therapeutic genes into neural cells for treatment of CNS disease. However, the transduction efficiency (TE) of rAAV2 in neural cells is not optimal for most therapeutic needs, at least in part because the single-stranded viral DNA must be converted to a double-stranded form prior to transgene expression. Several studies have demonstrated that rAAV2 is better able to transduce cells that have proliferation potential; for example, rAAV2 showed a 200-fold difference in transducing S-phase cells versus non-S-phase cells. Thus, researchers have sought to identify chemicals capable of modulating the transduction environment inside rAAV-infected cells at non-cytotoxic levels. Two DNA synthesis inhibitors, hydroxyurea (HU) and aphidicolin (Aphi), were shown to dramatically increase the transduction efficiency of rAAV in a stationary culture, and tyrphostin-1 (Tyr-1), an EGFR tyrosine kinase inhibitor, was shown to act as an extremely strong stimulator of rAAV transduction in multiple cell types. Recently, we showed that co-treatment with HU and Tyr-1 dramatically increased the transduction efficiency of rAAV2 in human cancer cells of different origins. Here, we investigated whether these same chemical co-treatments could enhance rAAV2 transduction efficiency in primary cortical neural cells.

Methods
Cell culture and virus preparation. 293T and SK-Hep1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (50 µg/ml), in a 5% CO2 incubator at 37 ºC. Primary cultures of mouse cortical neurons from 16 day-old Balb/c mouse embryos were prepared and maintained as described previously. Plasmid rAAV2-GFP, consisting of a GFP gene expression cassette embedded in the 5' and 3' terminal repeat sequences (TRs) of human AAV2 was generated with an AAV helper-free system (Stratagene, WA, USA). Briefly, 293T cells were transfected with the calcium phosphate method in a 1:1:1 molar ratio. The virus was purified using the calcium phosphate method in a 1:1:1 molar ratio. The virus was purified by the calcium phosphate method and harvested 2 days post-transfection, as described previously. The number of total rAAV2 particles in each preparation was estimated by ELISA.

Light and fluorescence microscopy
At various time points, cells were examined under a light microscope (LM) or a fluorescent microscope (FM) at 100-200 X magnification. Pictures were taken using a digital camera, and images were adjusted using the Adobe program to yield identical conditions for each experiment.

Cell cycle analysis. The change in DNA content was estimated by DNA staining with propidium iodide (PI). About 1-5 x 10^5 cells resuspended in PBS were treated with ice-cold 70% EtOH. After centrifugation, the cells were incubated in PBS with 0.1 mg/ml of RNase and 40 µg/ml of PI solution for 30 min at 37ºC. Flow cytometric analysis was carried out using a FACSCalibur flow cytometer (Becton Dickinson) and the DNA content was analyzed with the ModFit LT2.0 and Cellquest software packages.

Immunoblotting analysis and immunocytochemistry For the immunoblotting analysis, 1 x 10^5 cells were lysed. The lysate was pre-cleared and
resolved by 10% reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were immunoblotted using PVDF membrane and analyzed with 1-5 µg of primary antibody specifically recognizing pp38 (Cell Signaling Technology, MA, USA) or actin (Sigma, MO, USA). Bands were visualized by ECL analysis. For immunocytochemistry, cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h or absolute methanol for 30 min (Ki-67, Sigma), respectively. Each sample was then treated with a blocking solution containing 1% BSA and 0.1% TritonX100 in PBS, followed by the addition of the appropriate primary antibody (MAP II, Chemicon, Temecula, CA; GFAP, Chemicon; neurofilament; Sternberger) Finally, cells were treated with the appropriate Cy3- or FITC-conjugated secondary antibody and visualized under a fluorescence microscope.

Discussion

Light microscopic analysis showed that the routinely prepared primary cortical neural cultures contained cells with the typical morphological properties of neurons, including oval-shaped cell bodies and numerous dendritic stretches (Fig. 1A). Immunocytochemical observations indicated that the cell cultures consisted of mostly neural cells with minor contaminating astrocytes (Fig. 1A, MAP II and GFAP pictures). Four days after plating, the astrocyte population comprised 1-3 % of the culture; the population increased by several fold by day 11 under culture conditions that did not favor astrocyte proliferation (data not shown). The pattern of transduction by rAAV2 was examined by infecting the cells with 10^3-10^4 total rAAV2-GFP particles (Fig. 1B) per cell (TP/cell) and immunocytostaining for either neurofilament (neuron marker) or GFAP (astrocyte marker). Our results showed that the GFP signal was weakly visible at 2-3 day post-infection (dpi) and could be readily observed at 7 dpi (Fig. 1C). All GFP-expressing cells were neural cells, not astrocytes, in agreement with the previous reports 1,3,12. We then examined the response of these cultures to co-treatment with chemicals previously reported to dramatically increase the transduction efficiency (TE) of rAAV2 8,10.

Fig 1: Neural cells were preferentially transduced by rAAV2-GFP. Cells were isolated from the cortices of 16 day-old mouse embryos and plated. On day 4, cells were immunostained using antibodies specific to markers of neurons (MAP II) or astrocytes (GFAP). The results revealed that the neural cultures were healthy and relatively pure (A). The rAAV2-GFP expression vector consisted of a GFP expression cassette (in place of the viral rep and cap genes) under the control of the CMV promoter (B). Four days after plating, cells were infected with 103 TP/cell of rAAV2-GFP. GFP expression was analyzed at 7 day post-infection (dpi), and the results indicated preferential transduction of neural cells (C). LM, light microscopy; MAP II, GFAP or NF, immunostained with antibodies against MAP II, GFAP, or neurofilaments, respectively; GFP, examined for GFP signal; Merge, GFP + NF signals.
Our group and others have reported that Aphi or HU pretreatment and subsequent removal activates DNA polymerase and induces DNA synthesis. Under these conditions, stationary non-neural cells were able to reenter the cell cycle after G1/G0 arrest, resulting in a sudden surge of the S/G2/M phase population. Here, we evaluated whether the same phenomenon occurred in cultured cortical neural cells from 16 day-old mouse embryos, using cell cycle analysis with PI staining and immunocytochemistry for anti-Ki-67-positive nuclei. We found that most of the neural cells remained in G1/G0 stage (94.5 ± 1.6 %) on days 3 and 7 following Aphi or HU pretreatment (Fig. 2A, B). Consistent with the previous report, human hepatocellular carcinoma SK-Hep1 cells experienced a drastic increase in TE under these conditions, indicating that they had effectively escaped from G1/G0 arrest. The Ki-67 staining analysis revealed that only a low percentage of nuclei stained positively after HU pretreatment (Fig. 2C), and double-staining with GFAP indicated that the Ki-67 positive cells were astrocytes. Similar Ki-67 staining results were observed following Aphi pretreatment (data not shown).

Previous studies have shown that Tyr-1 can enhance the TE of rAAV2 by enhancing conversion of the rAAV genome from single-stranded to double-stranded. Tyr-1 can also increase transcriptional level of linear vector DNA templates by stimulating the activity of JNK and p38, as shown by increased phosphorylation of p38. Accordingly, we next examined whether p38 was phosphorylated in neural cells after Tyr-1 treatment. Our data
indicated that unlike SK-Hep1 cells, neural cells did not show any change in p38 phosphorylation status following treatment with Tyr-1 (Fig. 3).

Fig 3: Tyrphostin-1 treatment did not induce phosphorylation of p38 in the cultured neural cells. Immediately after Tyr-1 treatment, the p38 phosphorylation status was examined by Western blotting with an anti-phospho p38 antibody. Anti-actin was used as the loading control. The results indicate that p38 was not phosphorylated in neural cells in the absence (−) or presence (+) of Tyr-1.

Finally, we tested the enhancing effect on TE by pretreatment of the chemicals. The relative TE measured on 7 dpi indicated that the co-treatments had no positive effect on the neural cultures (Fig. 4). The chemical treatments did not induce any cytopathic effects in the neural cells as tested by MTT and LDH cell viability assays (data not shown), indicating that this ineffectiveness was not associated with cytotoxicity.

Fig 4: Effect of various chemical treatments on the transduction efficiency of rAAV2 in primary neural cells. Neural cells were pretreated with aphidicolin (Aphi, 5 µg/ml) or hydroxyurea (HU, 4 mM) for 16 h, tyrphostin-1 (Tyr-1, 125 µM) for 3 h, or combinations of these agents. The chemicals were then removed and cells were immediately infected with rAAV2-GFP at 5 x 10⁵ TP/cell. The relative transduction efficiency was evaluated by calculating the relative ratio of GFP-expressing cells versus total cells from 5 randomly selected microscopic fields. The results were compared to those of a control group (Control) that received no chemical treatment prior to rAAV2-GFP infection.
One of the critical issues for the practical usefulness of rAAV2 is its ability to direct sufficient transgene delivery to the proper target \(^{14}\). Our results showed that the transduction ability of simple rAAV2 infection in primary neural cells is quite poor (0.06 ± 0.02 % with 5 x 10\(^3\) TP/cell). This poor efficiency can be improved somewhat by co-infecting cells with wild-type adenovirus \(^5\) (data not shown) \(^{15,16}\), but this strategy is impractical, as target cells are eventually killed by adenovirus-induced cytopathic effects. Nevertheless, this property suggests that rAAV2 effectively enters primary cortical neural cells through virus-receptor interaction, but fails to express the transgene owing to other rate-limiting step(s) \(^{7,14}\). Previous studies have identified several chemicals capable of enhancing the rAAV2 TE by in various cell types, without attendant cytotoxicity \(^{8,16,17}\). However, our results show that, unlike the case in the previously tested cell types, neither DNA synthesis blockers (HU or Aphi) at low concentration nor EGFR tyrosine kinase inhibitor (Tyr-1) enhanced the TE in primary cortical neural cells of 16-day old mouse embryos. In non-neural cells, HU or Aphi pretreatment and subsequent removal leads to temporary synchronization at G1/G0, followed by synthesis of DNA polymerase and entry of cells into S phase, leading to efficient rAAV transduction \(^8,11\). In contrast, nearly 95% of the cortical neural cells in our cultures were already in G1/G0 stage, and no alteration in cell cycle was observed following treatment with the tested agents. Furthermore, the 16 day-old cortical neural cells did not show stimulation of p38 activity in the presence of Tyr-1, which may explain why Tyr-1 treatment had no positive effect on the TE. Together, these data indicate that the tested cortical neural cells may be locked in G0 stage and are unable to induce DNA polymerase synthesis. Thus, Hu, Aphi or Tyr-1 may only improve rAAV transduction in cells that retain their proliferation potential.

In sum, we herein showed that pretreatment of DNA synthesis and/or EGFR tyrosine kinase inhibitor does not enhance rAAV-mediated gene expression of 16-old cortical neural cells. When we examined cortical neural cells at an earlier stage (from 14 day-old mouse embryos), we detected around 11% of cells in S phase, as compared to only 4% in 16 day-old cells (Fig. 3A, 14D). This may suggest that a higher rAAV2 TE (with or without chemicals) could be achieved in cortical neural cells taken from an earlier stage of development. Recently, two groups reported the development of a self-complementary rAAV2 (scAAV2), which bypasses the host cell-mediated synthesis of the double-stranded rAAV genome from the single-stranded version, and consequently does not require target cells to have proliferating potential \(^{18,19}\). This new strategy may be useful for effective transduction of non-proliferating cortical neural cells at any stage, which possibility is under investigation.

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