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Cytokine Gene Delivery into the Central Nervous System Using Intrathecally Injected Nonreplicative Viral Vectors

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1. Introduction

The delivery of drugs through the bloodstream in patients affected by central nervous system (CNS)-confined multifocal diseases can be therapeutically ineffective because of the presence of the blood–brain barrier (BBB), which forms an inaccessible wall to the majority of CNS-targeting molecules. The BBB is a specialized endothelial structure formed by the interaction between endothelial cells and astrocytes. It can be distinguished from the normal endothelium for the presence of tight junctions between endothelial cells, which are impermeable to macromolecules and even ions, and for the reduced endocytic activity, which considerably decreases the number of molecules that can cross the BBB in a nonspecific fashion ***(1)***. Only the presence of specific transport mechanisms assures that molecules essential for the brain metabolism (e.g., amino acids and glucose) reach the brain parenchyma.

1.1. CNS Drug Delivery

Chronic inflammatory demyelinating diseases of the CNS, such as multiple sclerosis (MS), might benefit from anti-inflammatory therapies ***(2)***. However, promising treatments such as those based on the systemic administration of anti-inflammatory cytokines did not result in a consistent therapeutic effect in MS patients ***(3)***. The scarce capacity of cytokines to cross the BBB, along with their short half-life and autocrine/paracrine activity, might render necessary

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the delivery of these molecules directly into the CNS. Biological and physical vectors engineered with heterologous genes coding for anti-inflammatory cytokines might represent the appropriate tool to deliver cytokine into the CNS ***(4)***. Results obtained in rodents affected by experimental autoimmune encephalomyelitis (EAE), the animal models of MS, support this working hypothesis ***(5)***.

1.2 The Ependymal Way to Access the CNS

Here, we summarize the technical procedure and the troubleshooting of a novel strategy we recently established to access the CNS using viral vectors engineered with heterologous genes coding for anti-inflammatory cytokines. This approach is based on the injection into the cerebrospinal fluid (CSF) space trough the cisterna magna (i.c.) of nonreplicative viral vectors (**Fig. 1a**). Injected vectors, in turn, infect exclusively neuroectodermal cells lining the CSF space (including the Virchow–Robin spaces) and forming the blood–CSF barrier surrounding both the brain and the spinal cord (i.e., ependymal, choroidal, and leptomeningeal cells) (**Fig. 1b**). The viral genome enters into the nucleus of infected cells and dictates heterologous gene transcription (**Fig. 1c**). The protein coded by the transgene is then translated into the cell cytoplasm and secreted into the CSF (**Fig. 1d**). Secreted proteins diffuse, via the ependymal layer or the pia mater, into the CNS parenchyma, where they are still biologically active and can exert therapeutic activity.

Only vectors fulfilling the following criteria can be used. (1) vectors able to infect nondividing cells because ependymal and leptomeningeal cells cycle at a very slow rate; (2) vectors that can be obtained at very high titers because only very small volumes (up to 10 μL in mice) can be injected; (3) vectors with very low or no immunogenicity because the protocol is designed to interfere with an already ongoing immune reaction; (4) vectors expressing the transgene for the long term because repeated intrathecal injection of the vectors is a not feasible approach in a routine clinical setting.

Several viral vectors fulfill the above-mentioned criteria, among which herpes simplex virus (HSV) type-1-derived vectors, adenoviral vectors (AD), adeno-associated viral vectors (AAV), and lentiviral vectors. However, only HSV-1 and AD vectors, to our knowledge, have been intrathecally delivered, so far.

1.2.1. Herpes Simplex type-1-Derived Vectors

Several features make HSV-1 a likely candidate as a vector for gene transfer: (1) At least one-third of the 152 kb of the HSV-1 genome is made of genes nonessential for replication. These genes can be deleted and substituted by exogenous genes without any detrimental effect for in vitro viral growth. (2) HSV-1 can be easily propagated in several different cell lines (e.g.,

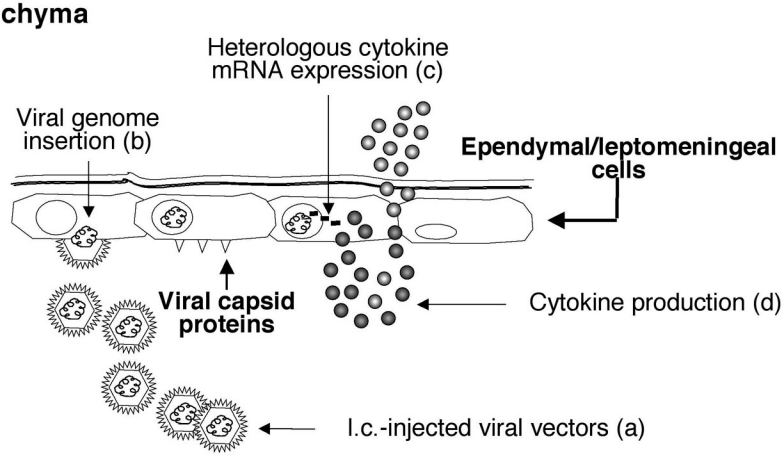

Fig. 1. The ependymal route. Nonreplicative viral vectors engineered to contain a cytokine gene are injected intracisternally in cerebrospinal fluid spaces (a); these vectors insert their genome into the cells lining ventricles and sub-arachnoid spaces (ependymal and leptomeningeal cells) (b) and induce them to transcribe (c) and translate the cytokine gene which is then released (d) into the cerebrospinal fluid. From there the exogenously produced cytokine can travel through the ependymal cell layer into the brain parenchima and exert there its potentially beneficial effect.

complementing cell lines), allowing the generation of high-titer viral stocks. (3) HSV-1 is able to infect several different cell types, regardless of the cell cycle, with high efficiency, making it an ideal candidate vector for several applications (i.e., infection of postmitotic neurons). (4) During the lytic cycle, many HSV-1 genes are expressed with high efficiency. A heterologous gene driven by a viral promoter can, therefore, produce large amounts of protein. (5) HSV-1 is able to persist in a state of latency for the whole life of its host. During latency, the viral genome is circularized and remains as an episome in the cell nucleus. Lytic genes are silent and only latency-specific transcripts are present. The introduction of foreign sequences under the control of latency-specific promoters may allow long-term transcription of transgenes.

We have been working with nonreplicative deletion mutant HSV-1 vectors. These deletions lead to the inability to replicate in normal conditions and lower their cytotoxicity. Immediate–early genes are the main target of the mutations because these genes are both essential for viral replication and responsible for most of the cytopathicity. Immediate–early genes are ICP infected cell

polypeptides ICP0, ICP22, ICP4, ICP27, and ICP47, in order of decreasing toxicity. Among those, only ICP4 and ICP27 are essential for replication, although the lack of some of the others (ICP0, ICP22) produces a marked decrease in viral titers. HSV-1 deletion mutants have been generated lacking three immediate–early genes (ICP4, ICP27, and ICP22) ***(6,7)*** and are propagated on a corresponding complementary cell line producing ICP4 and ICP27 (ICP22 is nonessential for in vitro viral growth). Using these herpetic vectors engineered to express interleukin (IL)-4, interferon (IFN)-γ, and FGF-II, we have obtained encouraging results in both mice and nonhuman primates affected by EAE ***(5,8–13)***.

1.2.2. Adenoviral Vectors

Adenoviruses have a double-stranded DNA genome of about 35–40 kb and lack an envelope. About 50 serotypes have been described, most of which causing, in humans, benign diseases of the respiratory tract. Serotypes 2 and 5 are the most studied for the obtainment of gene therapy vectors. In the adenoviral genome, between two inverted repeats (ITR) functioning as origin of replication, there are complex transcriptional units that can be divided into four early (E) and five late (L) regions. Of those, region E1A, the first expressed after infection, is essential for viral replication. First-generation vectors have been obtained deleting the E1 gene and replacing it with a 8- to 5-kb transgene expression cassette. The E1 gene product was provided in trans by a complementing cell line. Because of the high toxicity and immunogenicity of these vectors, high-capacity (HC), also named helper-dependent (HD) vectors, almost completely devoid of viral sequences, have been developed. HD vectors, which depend on an helper virus for in vitro growth, have only the two ITRs and the packaging signal and are, therefore, able to accomodate up to 30–35 kb of exogenous DNA. Contaminating helper virus is eliminated both by CRE-mediated excision of the packaging signal from its genome and, subsequently, by gradient purification. Main features of these vectors are (1) the ability to infect many different cell types regardless of the cell cycle (including postmytotic cells), (2) reduced toxicity and immunogenicity, and (3) long-term expression of the transgene (up to 6 mo). The genome can be entirely manipulated as a plasmid and grown in bacteria, making its genetic engineering easier. Adenoviral vectors have been used to infect ependymal–leptomeningeal cells, also in nonhuman primates ***(14–17)***.

1.3. Injection of HSV-1 Vectors into the Cisterna Magna to Access the CNS

To access the CSF space of mice, the most common procedure is an intraventricular injection performed using a stereotactic apparatus. This

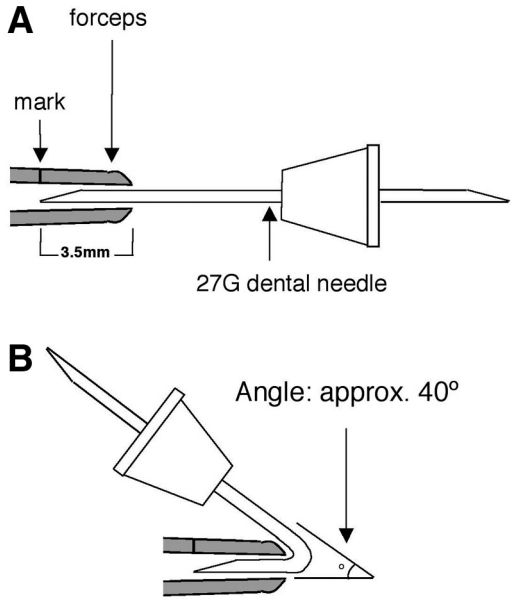

Fig. 2. Needle preparation. Hold the needle tightly with the tip at 3.5 mm from the end of the forceps **(A)**; bend the needle with the forceps at an angle of approx 40°, keeping the cutting edge inside **(B)**.

procedure is, however, time-consuming and limits the number of mice that can be treated in a single experiment. Here, we describe the application of a quick and simple intracisternal (i.c.) injection technique that can be used to deliver cytokine genes within the CNS. We also show a method to sample CSF from mice that represents an essential corollary technique to verify the efficiency of heterologous protein production within the CNS.

2. Materials

2.1. Injection Procedure

1. Flat forceps.
2. Dental needle 27G × 13/16 in. (0.40 × 21 mm) (*see* **Fig. 2**).
3. Bunsen burner.
4. Polyethylene tubing; inner diameter = 0.38 mm (0.015 in.); outer diameter = 1.09 mm (0.043 in.) (Becton Dickinson, cat. no. 427406).
5. 10-µL Hamilton GC syringe.
6. Diethyl ether.
7. Multichannel pipet reservoir.
8. Pipet tip box.

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